EFFECTS OF MATERNAL SEPARATION ON ALZHEIMER'S DISEASE-RELATED PATHOLOGY IN ADULT C57BL/6J MICE

by

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CHAPTER 1: GENERAL INTRODUCTION

It is estimated that 5.7 million people in the United States are afflicted with Alzheimer's disease (AD). AD is the only of the six leading causes of death that increased between the years 2000–2015. Deaths from AD increased 123% during that time. Further, the number of new cases of AD and other dementias annually is projected to double by 2050; the estimated disease prevalence is expected to reach nearly 14 million by 2050. ("2018 Alzheimer's Disease Facts and Figures," 2018). Moreover, AD is most devastating because of its impact on individuals suffering from the disease and those who care for them on a personal level. Health care payments for AD and other dementias are estimated at 277 billion dollars for 2018. Nearly 4 in 10 people who are providing unpaid care for a someone with AD report difficulty affording food and eating less as a result of caregiving ("2018 Alzheimer's Disease Facts and Figures," 2018). Most devastating is that the nature of the disease is such that patients experience significant cognitive deficits, memory loss, personality changes, and ultimately death (Huang & Mucke, 2012). The patients' health decline as the brain undergoes deterioration that is eventually fatal. This process is a devastating one, both for those suffering from AD as well as for the loved ones subject to witnessing this mental and physical decline.

Despite the ubiquity of Alzheimer's disease, its causes have yet to be fully elucidated despite sustained research efforts of labs across several disciplines (Selkoe, Mandelkow, & Holtzman 2012). Alois Alzheimer noted tangled fibrils in the place of neurons, and discovered decreased brain volume when he autopsied the brain of the first patient of Alzheimer's disease (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995). This decrease was caused, at least partly, by what came to be known as the two pathological hallmarks of AD, the accumulation of amyloid beta (Aβ) plaques (Glenner & Wong, 1984a, b) and neurofibrillary tangles (Grundke-

Iqbal et al., 1986). These pathologies are involved in both types of AD: Sporadic AD, the more common variant, is diagnosed after the age of 65, and develops relatively later in life. The other type, early-onset or familial Alzheimer's, is diagnosed before the age of 65. Early-onset AD has known genetic defects that affect the genes involved in the production of amyloid beta. Although sporadic AD is much more prevalent, its etiology remains unknown, though it likely involves several minor genetic mutations that increase the likelihood of developing the disease. The rising pervasiveness of AD combined with the lack of a treatment or cure present a compelling case for the necessity of further research.

The role of amyloid-beta in AD

Two key pathological hallmarks of Alzheimer's disease have been identified by scientists as amyloid beta (Aβ) plaques (Glenner & Wong, 1984a, b) and neurofibrillary tangles comprised of hyperphosphorylated tau, the microtubule associated protein that stabilizes the cytoskeleton of neurons (Grundke-Iqbal et al., 1986). Of the two, the role of Aβ in AD has been explored much more thoroughly by scientists. Aβ is formed by the proteolytic cleavage of amyloid precursor protein (APP) by the enzymes β-secretase and γ-secretase. APP is first cleaved by either α-secretase or β-secretase. Then both cleavage products are cleaved by γ-secretase. β-secretase cleavage of APP results in an N-terminal fragment, sAPP-β, and a C-terminal fragment (CTF) containing Aβ, β-CTF. The β-CTF is subsequently cleaved by γ-secretase resulting in a γ-CTF and $A\beta_{1\text{-}42}$, which is secreted through the cell membrane and then aggregates (Rezai-Zadeh et al., 2005; Haass & Selkoe, 1993). Alternatively, cleavage of APP by α-secretase results in the N-terminal fragment soluble APPα (sAPPα) as well as α-CTF in the middle of the Aβ domain. Because of this, subsequent cleavage of the fragment by γ-secretase does not result in the formation of toxic Aβ (Rezai-Zadeh et al., 2005).

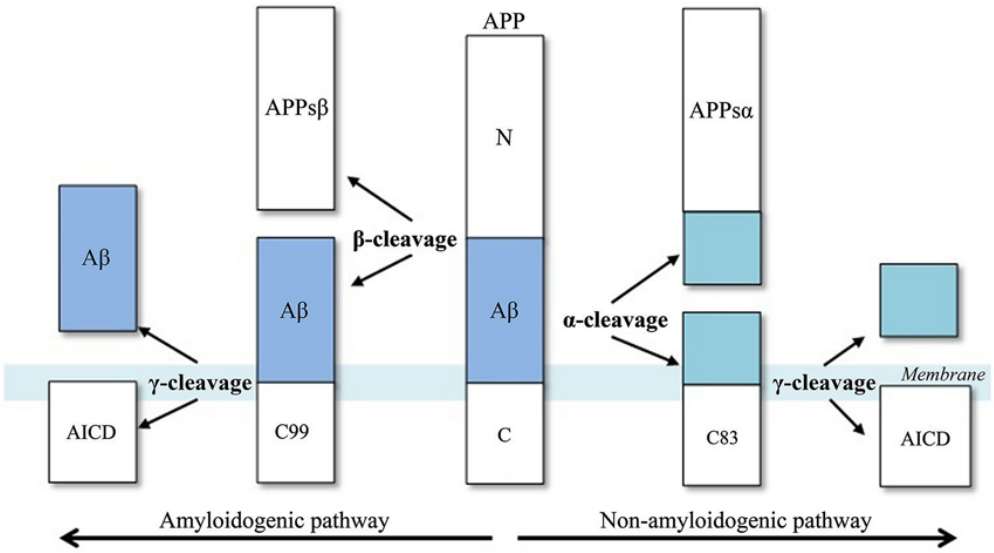

Figure 1. Cleavage of APP into Aβ. (Menting & Claassen, 2014).

The genetic causes of early-onset Alzheimer's disease have been identified as mutations in APP and the presenilin (PS)1 and (PS)2 genes of the γ-secretase complex, and these mutations initiate the amyloid cascade (Hardy & Higgens, 1992; Sherrington et al., 1995). These three genes mutate to alter the processing of APP, increasing amyloidogenic processing of APP, such that there is increased production of the toxic $A\beta_{1\text{-}42}$ (Bertram et al., 2010; Huang & Mucke, 2012; Seiffert et al., 2000) and the result is early-onset, autosomal dominant AD, though this accounts for fewer than 10% of Alzheimer's disease cases (Campion et al., 1999; Huang & Mucke, 2012). APP proteolysis is principal to the formation of Aβ, and thus β and γ secretase are frequently targets for AD interventions. Numerous animal studies have been successful decreasing or inhibiting β and γ secretases. γ-secretase inhibitors can block Aβ formation by binding to PS1 and PS2 and inhibiting the γ-secretase complex (Seiffert et al., 2000). Furthermore, decreasing levels of γ-secretase activating protein (GSAP) has also been shown to prevent the accumulation of Aβ through a reduction in γ-secretase activity (He et al., 2010). Research from our laboratory has demonstrated that imatinib methanesulfonate, a drug that

prevents GSAP from interacting with γ-secretase, prevented the elevation of hippocampal Aβ and the resultant cognitive impairment that follows LPS administration (Weintraub et al., 2013). Also, increased cerebrospinal fluid (CSF) levels of the main β-secretase, BACE1, has been postulated as an initial indicator of sporadic AD and a potential early intervention target (Evin, Barakat, and Masters, 2010). In an AD transgenic mouse, a reduction in BACE decreased the number of Aβ plaques and rescued cognition (Ohno et al., 2004). However, no interventions targeting secretases have proven successful in clinical trials (Schor, 2011).

Neurodegenerative effects of amyloid-beta

Aβ fibrils aggregate and form plaques, but soluble Aβ dimers, trimers, and oligomers are also pathogenic (Huang & Mucke, 2012). Researchers have long examined the role of Aβ plaques in AD, but recent research has shifted toward investigating the role Aβ oligomers might play, as patients with high plaque burden can lack cognitive symptomology (Erten-Lyons et al., 2009). Transgenic mice presenting with plaques have demonstrated no memory impairment during a period of augmented plaque formation that results in fewer oligomers, which is indicative of the detrimental role of oligomers potentially superseding that of plaques (Lesné, Kotilinek, & Ashe, 2008). Aβ oligomers lead to aberrant signaling and synaptic depression, through inducing excitotoxicity in glutamatergic neurons while impairing inhibitory interneurons (Palop & Mucke, 2010). In the presence of Aβ oligomers, sixty percent of excitatory synapses are lost (Mucke & Selkoe, 2012). Hippocampal and neocortical cholinergic, glutamatergic, and serotonergic synapses are most affected (Grutzendler & Morris, 2001). This Aβ-induced synaptic neuronal loss drastically impact learning and memory; subsequent deficits such as short-term memory impairment are present early in those afflicted with AD (Masters & Selkoe, 2012; Mayeux & Stern, 2012).

A significant impact of Aβ has been demonstrated through the research examining the effects of Aβ on long-term potentiation (LTP) in neuronal slice cultures. LTP occurs when there is high-frequency firing of action potentials, causing physiological changes to the postsynaptic neuron that strengthen a synapse and create more synapses. This can occur through the phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) channels and the insertion of new AMPA channels in the membrane (Mucke & Selkoe, 2012). This is thought to facilitate learning and memory. However, Aβ oligomers inhibit LTP. Aβ oligomers also increase long term depression (LTD) and synapse loss (Ittner & Götz, 2011; Mucke & Selkoe, 2012; Snyder et al., 2005), through promoting the endocytosis of N-methyl-D-aspartate (NMDA) receptors and decreased signaling to cAMP response element binding protein (CREB). CREB is a transcription factor that neurons need to survive (Snyder et al., 2005; Ittner & Götz, 2011). Through these means, Aβ results in substantial deficits to learning and memory.

Mouse models of AD

Numerous transgenic mouse models of AD have been developed to study the pathogenesis of early-onset AD, because of its genetic basis. The mouse models of familial AD overexpress one or more human genes that include mutations commonly found in humans with early-onset AD, APP, PS1, or PS2. These genetic mutations result in considerable elevations in Aβ production and plaque formation atypical of rodents. Also, transgenic mice with human APP, PS1, or PS2 mutations demonstrate significant cognitive impairments (Duff et al., 1996). One such type of transgenic mice, Tg2576 mice, have a genetic mutation such that results in the overexpression of APP and thus elevated $A\beta_{1-42}$ and plaque load in addition to impaired cognition by 9–10 months of age (Elder, Sosa, & Gasperi, 2010). The 5xFAD mice have a combination of 3 APP and 2 PS1 mutations, which results in substantially increased $A\beta_{1-42}$

production and plaque load that manifest early in life (Elder et al., 2010). The 5xFAD mice exhibit intraneuronal Aβ accumulation as well as plaque formation as early as 2 months of age (Oakley et al., 2006). Further, by 6 months of age 5xFAD mice demonstrate cognitive deficits in a contextual fear conditioning paradigm (Lison et al., 2014). However, sporadic AD is not modeled through the transgenic mice discussed above. Mouse models of familial AD are not ideal for studying sporadic AD because the mutations are in the genes known to cause early-onset AD. They also cause the pathology early on, making it difficult to study other factors that lead to the onset of pathology in sporadic AD. One factor known to contribute to pathology in early in the development of sporadic AD is inflammation.

Inflammation and AD

Research into what causes sporadic AD and what factors contribute to one's disease risk has been increasing, though no cause has been found. However, increasing numbers of labs are examining the role of inflammation in AD pathology, as research has indicated an role for inflammation in both AD risk as well as AD pathology in all stages of the disease. Markers of inflammation, or proinflammatory cytokines, are upregulated in the brains of AD patients (Kamer et al., 2008; McGeer & McGeer, 2001; Sokolova et al., 2008). Cytokines are small molecule messengers of the immune system that help organize an immune response, and are most often categorized as anti or proinflammatory. The function of the inflammatory response is typically adaptive, activated by a pathogen or tissue damage and facilitates the body's return to homeostasis (Medzhitov, 2008), and yet inflammation has been linked to diseases such as Alzheimer's (Van Eldik et al., 2016). In AD, there is a cyclical pattern that takes place, wherein proinflammatory cytokines up-regulate amyloidogenic APP cleavage (Lee et al., 2008) and this in turn leads to increased Aβ. Additionally, Aβ can activate microglial cells and lead to further

neuroinflammation (Meda et al., 1995; Schwab & McGeer, 2008). The link between increased inflammation and increased risk of AD has been supported through twin studies such as Engelhart et al., 2004 demonstrating multiple severe peripheral infections can accelerate the onset of AD pathology. Further, chronic inflammation through atopic disorders is also associated with increased risk for AD (Eriksson et al., 2008). Because of the connection between inflammation and AD, researchers have attempted to administer non-steroidal anti-inflammatory drugs (NSAIDs) to reduce AD pathology. NSAIDs inhibit cyclooxygenase (COX) activity, which can decrease the production of proinflammatory cytokines and restore cognition (Jain et al., 2002). However, NSAID clinical trials have yielded only modest benefits and have proved an ineffective AD intervention (Hall & Roberson, 2012).

One way researchers study inflammation is by administering lipopolysaccharide (LPS) to experimental subjects. LPS is a component of the cell wall of gram-negative bacteria, is recognized by peripheral macrophages, and activates toll like receptor-4 (TLR4), initiating the MyD88-dependent intercellular signaling cascade (Galanos et al., 1985; Wright et al., 1989). This induces an immune response that results in microglia and macrophages releasing several proinflammatory cytokines (Lu et al., 2008). Our lab has previously demonstrated that one intraperitoneal (i.p.) administration of LPS (250 μg/kg), a bacterial mimetic, stimulates an inflammatory response that includes significantly elevated levels of peripheral IL-1β, IL-6, and TNF-α, monocyte chemoattractant protein-1 (MCP)-1 and macrophage inflammatory protein (MIP)-1α that peak around 4 hours following administration in C57BL/6J mice (Kahn et al., 2012; Kranjac et al., 2012). We then extended this finding to examine the relationship between inflammation and Aβ by demonstrating that seven, once-daily i.p. injections of LPS (250 μg/kg) increases the amount of hippocampal Aβ and results in cognitive deficits (Kahn et al., 2012;

Kranjac et al., 2012). This Aβ is possibly produced in the brain, as sub-diaphragmatic vagal afferents can transduce cytokine signaling from the periphery and induce the production of central cytokines (Goehler et al., 1997; Laye et al., 1995). Another possibility is that the Aβ is produced in the periphery and transported into the brain. This is because LPS can compromise the blood brain barrier (BBB) tight junctions and allow larger molecules to pass through (Jaeger et al., 2009). Chen et al. (2008) found more microglia and proinflammatory cytokines in the hippocampi of old mice injected with LPS than young mice. A greater inflammatory response in older mice suggests that age plays a role in AD, potentially through differences in Aβ.

Stress and its impact on AD

Stress

Walter Cannon explained that much of biology fluctuates in response to the needs of a given situation (Cannon, 1914). He described homeostasis, a process of balancing physiological systems within tolerable activation limits, and referred to periods of increased arousal as the fight or flight response (Cannon, 1929). What constitutes a stressor is specific to the individual, and is influenced by factors such as learned associations, and the context in which the event occurred (Edwards, King, and Fray, 1999; Lazarus et al., 1965). In response to stress, in attempting to return to homeostasis, the body utilizes the hypothalamic-pituitary-adrenal (HPA) axis (Berton and Nestler, 2006; Chrousos, 2009). The HPA axis responds to stress by releasing corticotropin-releasing factor (CRF) from the hypothalamus (Rivier & Vale, 1983). CRF causes the anterior pituitary to release adrenocorticotropic hormone (ACTH), which results in the production of glucocorticoids by the adrenal cortex (Smith & Vale, 2006; Kloet & Derijk, 2004). Glucocorticoids have wide-ranging influence peripherally, including altering metabolism, the immune system, behavior, and the cardiovascular system. In order to enable the body to recover

from stress, there exists a negative feedback loop, wherein glucocorticoid receptors in the hippocampus bind circulating glucocorticoids and decrease further glucocorticoid release (Keller-Wood & Dallman, 1984; Smith & Vale, 2006; Jacobson & Sapolsky, 1991). Another essential component of this activation is signaling from the sympathetic nervous system (SNS) and the subsequent release of the catecholamines epinephrine and norepinephrine from the adrenal medulla (Roldan et al., 1974; Cannon, Britton, Lewis, & Groeneveld, 1927; Selye, 1946).

Stress affects numerous body systems, including inflammatory processes (Zlatković, & Filipović, 2013; Black, 1994). In response to an acute stressor, inflammation occurs through high mobilty group box-1 protein (HMGB-1)-mediated priming of the nucleotide-binding domain, leucine-rich repeat, pyrin domain containing protein 3 (NLRP3) inflammasome as well as increased pro-inflammatory cytokine production including IL-1β, TNF-α, and IL-6 (Walker, Nilsson, & Jones, 2013; Weber et al., 2015) and gliosis. Johnson et al. (2003) demonstrated that an extreme acute stressor, inescapable tail shocks, exacerbated the inflammatory response to LPS administration following the onset of the stressor, resulting in increased peripheral IL-1β, IL-6, and TNF-α. An extension of this work found that the proinflammatory effects were amplified utilizing a morning collection, a time when LPS-induced glucocorticoid production was significantly elevated compared to controls, as opposed to the evening collection (Johnson et al., 2003).

Conversely, chronic stress can result in immunosuppressive effects, characterized by a decrease in immue cell number, reduced natural killer (NK) cell cytotoxicity, and an increase in regulatory T cells (Dhabhar, 2009; Dhabhar & McEwen, 1997; Irwin et al., 1990). Additionally, the environment plays a significant role in the body's response to stress; the effects of stress

differ from person to person. One such example is the extent to which rat pups are groomed by their mothers alters their development, permanently impacting their adulthood physiological and behavioral response to stress (Liu et al., 1997; Caldji et al., 1998). Increased maternal care behaviors leads to pups that display decreased plasma ACTH and corticosterone in response to stress and a more sensitive glucocorticoid feedback loop later in life (Liu et al., 1997).

Stress and health

Temporary activation of the HPA axis allows the body to prepare for a stressor and then the aid the body's returns to homeostasis. However, prolonged activation of the HPA axis has been shown to be maladaptive (McEwen, & Gianaros, 2010). An increased number of stressors in adulthood can predict mortality (House, Landis, & Umberson, 1988), and produce effects similar to those of cardiovascular disease (CVD) and stroke in humans (Friedler, Crapser, & McCullough, 2015). Moreover, stress is a risk factor for CVD and ischemic strokes, and, while controlling for all other risk factors, increases the likelihood of re-infarction and mortality in patients (Friedler et al., 2015). Additionally, general emotional stress has also been shown to increase one's risk of developing type II diabetes (Pouwer, Kupper, & Adriaanse, 2010) in addition to making the existent disease worse (Moran et al., 2015). Type II diabetes as well as CVD result in an increased risk of developing sporadic AD (Haan, 2006). Further, depression also is exacerbated by stress and, in turn, negatively impacts AD progression (Rothman & Mattson, 2010; Kloet et al., 2004). Though the precise mechanisms for this are unknown, the connection between stress and immune function involves sympathetic fibers between the spinal cord and primary and secondary lymphoid tissues, for example the spleen and thymus (Felten & Felten, 1994). Also, many immune cells have catecholamine and glucocorticoid receptors,

activation of which can lead to gene activation, suppression of cellular signaling, and leukocyte trafficking (Padgett & Glaser, 2003).

Stress and AD

According to clinical data, stress can be a risk factor for Alzheimer's disease (Wilson et al., 2005; Friedler et al., 2015). The process of aging results in decreased HPA axis regulation and the hypersecretion of glucocorticoids in humans and rats, which potentiates hippocampal damage as a result of Aβ (Friedler et al., 2015). Further, rats that have experienced a form of early life stress where they are separated from their mothers as pups demonstrate increases in plasma corticosterone and decreased hippocampal glucocorticoid receptor density; this is indicative of depressed HPA axis feedback inhibition, and accompanies cognitive deficits and increased amyloidogenic processing of APP (Solas et al., 2010). Researchers also found that when mice experience various daily stressors across four weeks, the result is increased hippocampal β-CTF and BACE protein levels, which indicates increased amyloidgenic cleavage of APP by the β and γ secretases (Catania et al., 2009). Research also shows stress to exacerbate Aβ production in transgenic rodent models of AD (Devi et al., 2010; Lee et al., 2009; Dong et al., 2004). Dong et al. (2014) demonstrated that chronically isolated Tg2576 transgenic mice displayed increases in $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$ levels, plaques, and anxiety-like behaviors in elevated plus and spontaneous alteration behavioral paradigms. Further research found that APP/PS1 transgenic mice isolated for 4 months showed impaired spatial working memory and an increased $A\beta_{40}/A\beta_{42}$ ratio without plaque formation (Hsiao et al., 2011). However, this effect is not uniform, as 5xFAD transgenic mice administered 5 days of restraint stress, in which animals are immobilized, showed elevated hippocampal but not neocortical Aβ in female mice only. Additionally, illustrating the importance of the nature and duration of the stressor, TgCRND8

transgenic mice from 1 to 3 or 4 to 6 months of age subjected to restraint stress did not have a significant increase in Aβ plaque number (Yuan et al., 2013).

The numerous mechanisms through which stress increases Aβ have yet to be fully understood, and there appears to be an interaction between age at onset and duration of stress and its impacts on AD-related pathology. Studies show stress-induced cognitive deficits and neurodegeneration are corticotropin releasing factor 1 (CRF1) and corticotropin releasing factor receptor 1($CRFR_1$)-dependent (Carroll et al., 2011; Dong et al., 2014). The effects on Aβ may be beta-site APP cleaving enzyme 1 (BACE1)-dependent, as stress increases BACE1 expression in addition to decreasing methylation, thereby increasing gene expression, of 5'—C′—phosphate'—G'—3' (CpGs) of the BACE1 promoter (Cordner & Tamishiro, 2016). Hippocampal expression of BACE1 was correlated with adrenal weight, indicating that glucocorticoids may epigenetically alter BACE1 expression (Cordner & Tamishiro, 2016). Further, *in vitro* and *in vivo* glucocorticoid treatment increased levels of the precursor to Aβ resulting from BACE1 cleavage, CTF-β (Green et al., 2006). Additionally, there may be a role for oxidative stress in these processes, as chronic stress led to activation of nuclear factor kappa-B (NF-κB) and increased inducible nitric oxide synthase (iNOS) expression (Zlatkovic and Filipović, 2013). Further, the antioxidant N-acetylcysteine prevented isolation stress-induced increases in γ secretase and Aβ (Hsiao, Kuo, Chen, & Gean, 2012). Alternatively, gliosis induced by acute stress causes an increase in pro-inflammatory cytokines that can increase the Aβ that remains through chronic stress (Walker et al., 2013; Weber et al., 2015).

Early life stress and health

Adulthood stressors alter APP processing and exacerbate Aβ production in addition to causing cognitive deficits (Devi et al., 2010; Lee et al., 2009; Dong et al., 2004). The role of

early life stressors in the development of AD pathology later in life is less well known. Exposure to adverse environmental events early in life is thought to increase risk for developing neuropsychiatric conditions, including affective disorders and psychosis (Chapman et al. 2004; van Winkel et al. 2013). It has been shown that maternal separation, a form of early life stress where mice are separated from their mothers, causes a long-lasting disruption in HPA axis function that leads to increased basal levels of corticosterone in adult animals (Clarke, 1993; Slotten et al., 2006; Roque et al., 2014) as well as decreased thymic weight (Roque et al., 2014). Maternally separated animals also exhibit increased anxiety and depressive like behaviors in adulthood (Roque et al., 2014). Pinheiro et al. (2014) demonstrated that, in addition to inducing memory deficits, maternal separation increased the levels of IL-10 and TNF-a in the hippocampus and decreased hippocampal levels of brain derived neurotrophic factor (BDNF) in adult animals. BDNF is a neurotrophic factor that promotes neuronal survival, enhances synaptic plasticity and alters dendritic spine morphology (Yang et al., 2009).

The parameters surrounding early life stress determine the effects of the intervention and lead to differing developmental trajectories. Following the typical duration of maternal separation, dams are reported to increase maternal care immediately upon reunion, but long-term care is unchanged, indicating any lasting effects are due to the separation and not differential maternal care (Own & Patel, 2013). Early postnatal handling alone has been shown to decrease anxiety behaviors, and stress-induced corticosterone secretion in rats (Vallee et al., 1997), and delay the onset of cognitive impairment in transgenic AD mice (Lesuis et al., 2017). One recent hypothesis called the match mismatch, or maternal mismatch hypothesis, suggests that resilience in conferred when animals experience stress early in life and then are subject to a secondary stressor in adulthood (Santarelli et al., 2017). It argues these animals are better off than animals

just experiencing early life stress or stress in adulthood, displaying decreased anxiety and corticosterone (Santarelli et al., 2017, Biggio et al., 2014). However, a longer duration of early life stress, or early life stress alone often have detrimental effects on health outcomes in adulthood, as research has shown that a brief daily maternal separation of just 15 minutes from post natal day 1–21 results in adverse health outcomes in Balb/c mice, including dysregulating microglial function in the developing hippocampus (Delpech et al., 2016).

If stressors early in life impact stress responsiveness and cognition in adulthood (Liu et al., 1997; Caldji et al., 1998), it merits investigating whether early life stressors can also impact APP processing and Aβ production later in life. Rats that have undergone maternal separation display increased plasma corticosterone and decreased hippocampal glucocorticoid receptor density, indicating depressed HPA axis feedback inhibition, in addition to cognitive deficits and increased amyloidogenic processing of APP (Solas et al., 2010). A subsequent maternal separation study in rats found increased BACE1 expression as well as increases in hippocampal $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels (Martisova, Aisa, Guerenu, & Ramirez, 2013). Additionally, APP/PS1 mice that underwent bedding restriction early in life, a different early life stress paradigm that deprives the dam of nesting material, exhibited increased hippocampal $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels and cognitive deficits, as well as increased corticosterone and BACE1 (Lesuis et al., 2018a). Furthermore, Hoeijmakers et al. (2017) demonstrated bedding restricted APP/PS1 pups had increased Aβ plaque load by 10 months of age. By 9 months of age, APP/PS1 mice that had been maternally separated exhibited exacerbated cognitive deficits and more cortical and hippocampal Aβ plaques than non-stressed animals (Hui et al., 2017). A review by Lesuis et al., (2018b) indicates HPA axis alterations and priming of the neuroinflammatory response as potential mechanisms the modulation of AD neuropathology and cognition by early life stress. Taken

together, current research indicates that early life stress can impact adulthood AD pathology, but the effects of maternal separation have not been investigated fully in non-transgenic mice.

There also appear to be sex differences in the adulthood effects of maternal separation. For example, Slotten et al. (2006) found that MS affected the HPA axis, body weight, and locomotor activity more significantly in female rats, whereas males displayed a more significant cognitive deficit in T-maze acquisition. Other studies involving maternal separation have demonstrated that stress decreases threat memory in a sex specific manner, either in females (Kosten, Lee, & Kim, 2006), or in males alone (Lehmann, Pryce, Bettschen, & Feldon, 1999). Bedding restriction impaired context memory in female, but not male, mice (Manzano-Nieves, Gaillard, Gallo, & Bath, 2018). The ambiguous nature of the effects of maternal separation necessitates further research to determine how early life stress can alter cognition and the development of AD pathology later in life.

CHAPTER 2: THE EFFECTS OF MATERNAL SEPARATION AND 7 LPS INJECTIONS

Abbreviated Introduction

Research has demonstrated there are detrimental effects of maternal separation and early life stress on some AD-like markers, such as cognitive deficits and proinflammatory cytokines, in non-transgenic animals (Pinheiro et al., 2014; Martisova et al,. 2013; Solas et al., 2010). Investigators have also recently begun exploring the effects of maternal separation on Alzheimer's disease pathology in AD transgenic animals (Hui et al., 2017). However, the majority of AD cases are not early-onset, the type modeled by AD transgenic animals. In an attempt to investigate what might underlie sporadic AD, while studying the AD pathologies of Aβ and tau, researchers have developed an inflammation-based model. In order to investigate the relationship between maternal separation and AD-like pathology in adulthood, the present study set out to examine if maternal separation early in life would induce cognitive deficits in adulthood, and exacerbate hippocampal Aβ production, using such a model (Lee et al., 2008). Previous studies from our laboratory has demonstrated that one intraperitoneal (i.p.) administration of LPS (250 μg/kg), a bacterial mimetic, stimulates an inflammatory response that includes significantly elevated levels of peripheral IL-1β, IL-6, and TNF-α, monocyte chemoattractant protein-1 (MCP)-1 and macrophage inflammatory protein (MIP)-1α that peak around 4 hours following administration in C57BL/6J mice (Kahn et al., 2012; Kranjac et al., 2012). We then extended these findings to examine the relationship between inflammation and Aβ by demonstrating that seven, once-daily i.p. injections of LPS (250 μg/kg) increase hippocampal Aβ production and results in cognitive deficits (Kahn et al., 2012).

The present studies explore whether maternal separation and 7, once-daily injections interact to exacerbate AD-like pathology in non-transgenic mice. Our laboratory has previously

shown that repeated administrations of LPS induce inflammation, cause cognitive deficits, and increase Aβ levels in the central nervous system (Kahn et al., 2012). Since maternal separation can result in increased proinflammatory cytokine production and cognitive deficits in non-transgenic animals (Pinheiro et al., 2014), and increase AD-like pathology in AD transgenic mice (Hui et al., 2017), the potential interaction between MS and LPS administration during adulthood necessitates investigation. In order to explore a potential mechanistic effect, hippocampal BACE1 was also investigated. BACE1 is the main β-secretase responsible for the amyloidogenic cleavage of amyloid precursor protein along with γ-secretase that generates the toxic $A\beta_{1-42}$ fragment. Increased cleavage of APP by BACE1 and then γ-secretase as opposed to cleavage by α-secretase could be a mechanistic link to increased production of $A\beta_{1-42}$ (Selkoe, 1994). The purpose of the current project was to study the effects of early life stress and repeated bouts of inflammation on the development of AD-like pathologies in adulthood, and investigate potential sex specificity of these effects.

We hypothesized that an early life stressor, maternal separation, would significantly exacerbate LPS-induced soluble amyloid beta production and cognitive deficits in C57BL/6J mice in comparison to non-maternally separated animals. More specifically, we hypothesized that there would be a significant increase in amyloid beta in maternally separated, LPS-treated male and female animals over non-maternally separated and saline controls. It was further hypothesized that maternally separated animals with exacerbated Aβ production would have increased BACE1 expression as compared with non-maternally separated or saline animals. Additionally, it was hypothesized that maternally separated LPS-treated mice would perform worse in contextual fear conditioning (CFC), a hippocampus-dependent learning and memory task, than non-maternally separated or saline-treated animals. Freezing in CFC is indicative of

learning of the context shock association, and mice that remain active display a contextual learning deficit.

Methods

Subjects

Animals used in experiments were male C57Bl/6J mice bred in the TCU vivarium. All animals were housed and treated in accordance with protocols approved by Texas Christian University's Institutional Animal Care and Use Committee (IACUC) and with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). All subjects were housed in groups of 2 to 4 in standard polycarbonate cages (12.5cm x 15cm x 25cm). Food and water were available *ad libitum* and animals were maintained on a consistent light/dark schedule, with lights on at 0700 and lights off at 1900.

Treatment conditions

On post-natal day 2 (PND 2; Solas et al., 2010), pups from all litters were sexed, mixed, and randomly assigned to each dam (Roque at al., 2014). Each dam and the corresponding litter were randomly assigned to experimental conditions: Maternal Separation (MS) or Control (C). Maternally separated pups were separated from the dam for 3h daily from PND 2-21, and control pups remained with the dam till weaning at PND 23 (Solas et al., 2010) Maternally separated animals were transferred with nesting material and were monitored in an incubator to maintain constant temperature of 31°C. At weaning all animals were grouped by sex into groups of 3 or 4 and were allowed to age normally until adulthood. At 4–6 months of age, males were randomly assigned into the following treatment conditions: MS + LPS, MS + Saline, C + LPS, or C + Saline. Following group assignment, animals were given 7, once-daily injections of either 250μg/kg of LPS (*Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) or

Saline as a control. LPS was administered in a weight-dependent manner to ensure the dose met a concentration of 250 µg/kg. Twenty-four hours after the final injection, animals underwent a contextual fear-conditioning paradigm and then tissue was collected.

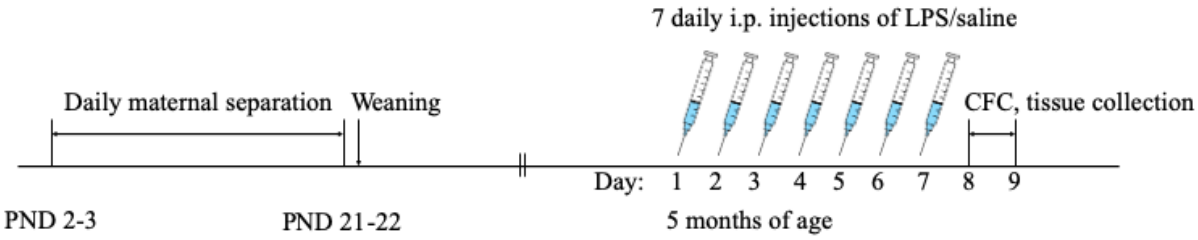

Figure 2. Experimental timeline: Experiment 1. MS animals were maternally separated from PND2-2 while non-MS animals were undisturbed, and then both groups aged until adulthood and then were administered 7, once-daily injections prior to CFC and tissue collection.

Behavioral Paradigm

After extended isolation or group housing, animals underwent contextual fear conditioning. This protocol includes a training session (day 1) and a testing session (day 2) 24 hours later. The training session consists of a 120s acclimation period followed by a 2s foot shock at 0.5mA, 60s with no shock, and then an additional 2s 0.5mA shock. Animals remain in the chamber for an additional 60s following the conclusion of the aversive stimulus. On testing day, animals are placed back into the chamber and freezing is monitored for 180s, but no shocks are delivered. Contextual fear conditioning is conducted in chambers (Coulbourn Instruments, Whitehall, PA, 7Wx7Dx12H) with an electrified grid floor through which the aversive stimulus is delivered. The delivery of the stimulus and the resulting freezing behavior is monitored and calculated using FreezeFrame™ software (ActiMetrics Software, Wilmette, IL).

Previous research from our laboratory has demonstrated that the incorporation of an olfactory cue (peppermint oil 1:10 in water) and a highly salient wall design (black polka dots) into the context increases the mouse's percent freezing. We interpret this increase in freezing

behavior as better learning of the context-shock pairing (Phillips & Ledoux, 1992; Kahn et al., 2012; Kranjac et al., 2012). The time freezing was analyzed using analysis of variance (ANOVA) procedures (SPSS 22.0, IBM, Armonk, NY), in which Genotype (5xFAD+/-) and Treatment (isolated/group housed) were used as independent variables. All statistical analyses were conducted using an alpha level of ≤0.05 to determine significant group differences. Following a significant omnibus F value, post-hoc comparisons utilized Fisher's PLSD.

Tissue preparation and ELISA

Tissue extraction

The brain was removed and the hippocampi were excised and the tissue homogenized with PRO-PREP (Bora Scientific, Boca Raton, FL). The samples were immediately frozen on dry ice and stored at -80 °C. The lysate was centrifuged at 10,000 rpm for 40 minutes, before the lysate was removed and a *DC* Protein Assay (Bio-Rad Laboratories, Hercules, CA.) conducted.

***DC* Protein Assay**

DC Protein Assays utilize a working reagent that is used with detergent-based buffers. The protein standard curve consists of dilutions from 0.2–1.5 mg/ml of γ-globulin, made in the same buffer as the lysates. Five µl of standards and 5 µl of sample were pipetted into a 96 well plate with 25 µl of reagent A' and 200 µl of reagent B. After 15 min, the plate was put into the plate reader (BMG LabTech FLUOstar Omega, Cary, NC), and the optical density of the samples was read at 750 nm. The results were then used to standardize general protein content for the ELISA.

Aβ ELISA procedure

The BetaMark $A\beta_X$-42 ELISA (BioLegend, San Diego, CA) was performed according to manufacturer instructions. In brief, the samples were diluted with working incubation buffer including the HRP- labeled detection antibody, and the dilutions of the standard were plated in

duplicate. Samples were diluted 1:4. The plate was incubated overnight at 2–8 °C. The next day, wells were washed five times with wash buffer and the TMB substrate was added to each well. The plate was then incubated for 45 min at room temperature and well optical density read at 620 nm (BMG LabTech FLUOstar Omega, Cary, NC).

Western blotting

Samples were diluted in sample buffer to a working concentration of 1 g/L and then denatured via boiling at 100◦ C for 5 m in the presence of β-mercaptoethanol (Sigma, St. Louis, MO) to reduce disulfide bonds. Proteins were separated by size with Mini-Protean® TGXTM precast gradient gels (4–15%; BioRad, Hercules, CA). 200 V was applied across the gel for 40 minutes, the gel was removed and equilibrated in Towbin transfer buffer. Then proteins were transferred onto a polyvinylidene fluoride (PVDF) (Millipore, Billerica, MA) membrane at 0.15 amps per gel for 40 minutes with a semi-dry transfer unit (BioRad). After transfer, membranes were blocked using 5% bovine serum albumin in Tris buffered saline with 0.3% Tween® 20 (TBST) for 2 hours. Primary antibodies (BACE1 1:750, β-actin 1:500) were diluted in TBST, applied to the membrane and incubated overnight at 4◦ C. The next day, membranes were washed in TBST and then peroxidase-conjugated AffiniPure secondary antibodies were applied (Jackson ImmunoResearch Laboritories, Inc., West Grove, PA) for 2h at room temperature. Membranes were washed and then coated with the detection reagent, SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Wiltham, MA). Chemiluminescence was imaged using a Syngene G:Box (Syngene, Fredrick, MD), and densitometry analysis was performed using GeneTools software (Syngene, Fredrick, MD). β-actin was used as the loading control.

Results

Experiment 1A: Effects of Maternal Separation and 7 LPS Injections on Cognition

Maternal separation leads to impaired freezing in contextual fear conditioning in males

A two-way analysis of variance (ANOVA) was used to determine whether maternal separation impacted cognition in a contextual fear conditioning paradigm. Analyses revealed a main effect of Condition (MS or no MS; $F(1, 29)= 4.219$, $p = .049$) such that MS animals froze significantly less in CFC than non-MS animals. There was not a significant main effect of Treatment (LPS or Saline; $F(1, 29)= .010$, *NS*) or a significant Condition x Treatment interaction ($F(1, 29)= 1.747$, *NS*; Figure 3). Overall, maternal separation led to impaired freezing in contextual fear conditioning.

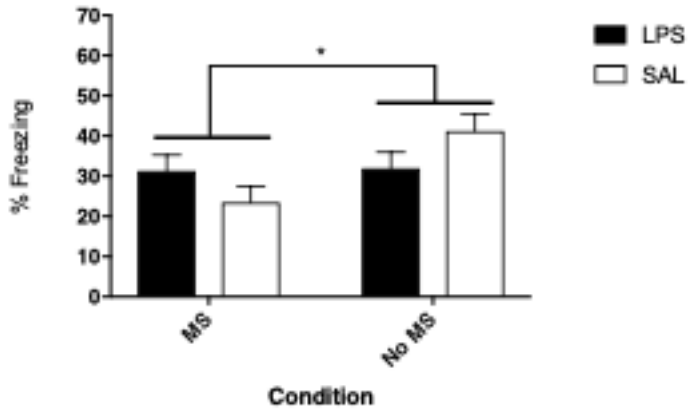

Figure 3. Maternal separation leads to impaired cognition in males. A 2x2 ANOVA revealed a main effect of condition, such that maternally separated animals froze significantly less than control animals regardless of treatment. Bars represent mean ± SEM. Significance differences ($p<0.05$) are designated by *.

Maternal separation did not lead to impaired freezing in contextual fear conditioning in females

A two-way analysis of variance (ANOVA) was used to determine whether maternal separation impacted cognition in a contextual fear conditioning paradigm. Analyses revealed

there was not a main effect of Condition (MS or no MS; $F(1, 56)= 2.317$, *NS*) or Treatment (LPS or Saline; $F(1, 56)= 1.359$, *NS*) or a significant Condition x Genotype interaction ($F(1, 56)= .358$, *NS*; Figure 4). Overall, maternal separation did not lead to impaired freezing in contextual fear conditioning.

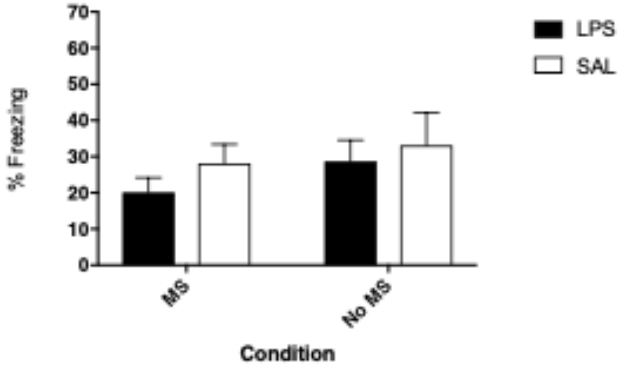

Figure 4. Maternal separation did not lead to impaired cognition in females. A 2x2 ANOVA revealed no significant main effects or interaction effects. Bars represent mean ± SEM. Significance differences ($p<0.05$) are designated by *.

Experiment 1B: Effects of Maternal Separation and 7 LPS Injections on Aβ

7 LPS injections significantly increase hippocampal Aβ levels in males

A 2x2 ANOVA was used to determine if maternal separation impacted hippocampal Aβ levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 29)$ 28.256, $p < .001$) such that LPS-treated animals had significantly more hippocampal Aβ than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 29)= 1.256$, *NS*) or a significant Condition x Treatment interaction ($F(1, 29)= .177$, *NS*); Figure 5). Overall, maternal separation did not significantly increase hippocampal Aβ levels.

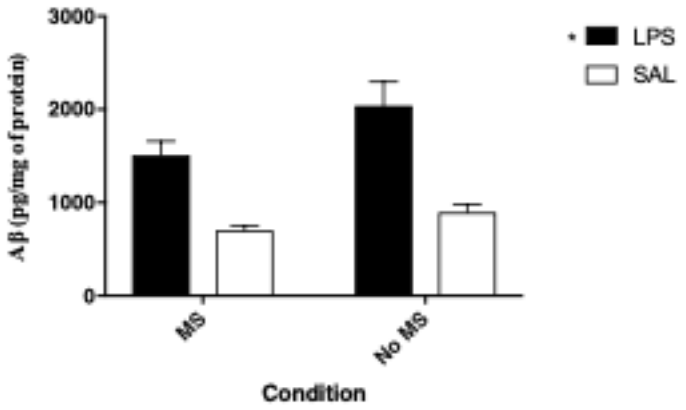

Figure 5. Maternal separation did not significantly impact soluble Aβ levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more hippocampal Aβ than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

7 LPS injections significantly increase hippocampal Aβ levels in females

A 2x2 ANOVA was used to determine if maternal separation impacted hippocampal Aβ levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 24)$ 12.512, $p = .002$) such that LPS-treated animals had significantly more hippocampal Aβ than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 24)= 2.306$, *NS*) or a significant Condition x Treatment interaction ($F(1, 24)= .205$, *NS*); Figure 6). Overall, maternal separation did not significantly increase hippocampal Aβ levels.

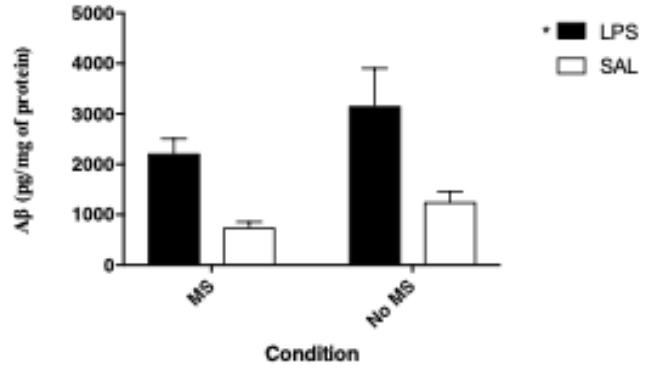

Figure 6. Maternal separation did not significantly impact soluble Aβ levels in females. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more

hippocampal Aβ than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

Experiment 1C: Effects of Maternal Separation and 7 LPS Injections on BACE1

7 LPS injections and maternal separation increase hippocampal BACE1 levels in males

A 2x2 ANOVA was used to determine if maternal separation impacted hippocampal BACE1 levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 21)= 8.849$, $p = .007$) such that LPS-treated animals had significantly more hippocampal BACE1 than saline-treated animals. There was a marginally significant main effect of Condition (MS or no MS; $F(1, 21)= 3.211$, $p = 0.088$), in which MS animals had more BACE1 than non-MS animals. There was not a significant Condition x Treatment interaction ($F(1, 21)= .186$, *NS*); Figure 7). Overall, maternal separation and 7, once-daily injections increased hippocampal BACE1 levels.

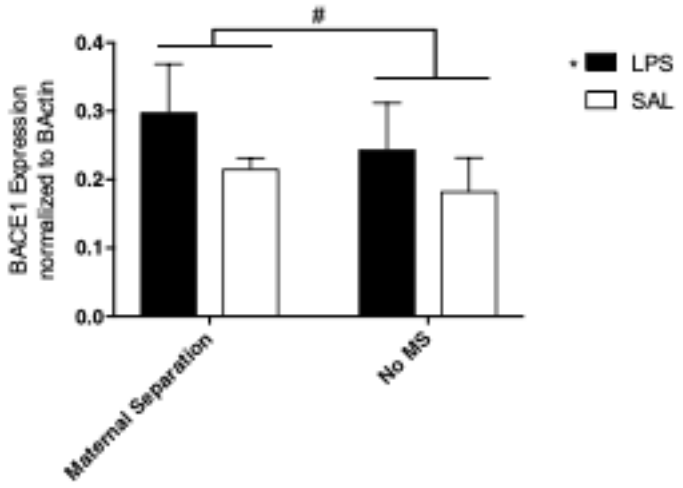

Figure 7. Maternal separation increased BACE1 levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more hippocampal BACE1 than saline animals and a marginally significant main effect of Condition such that MS animals had more BACE1 than non-MS animals. Significance differences (p<0.05) are designated by *. Marginally significance differences (p=0.088) are designated by #.

7 LPS injections significantly increase hippocampal BACE1 levels in females

A 2x2 ANOVA was used to determine if maternal separation impacted hippocampal BACE1 levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 25)$ 6.018, p = .021) such that LPS-treated animals had significantly more hippocampal BACE1 than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 25)$= 1.229, *NS*) or a significant Condition x Treatment interaction ($F(1, 25)$= .035, *NS*); Figure 8). Overall, maternal separation did not significantly increase hippocampal BACE1 levels.

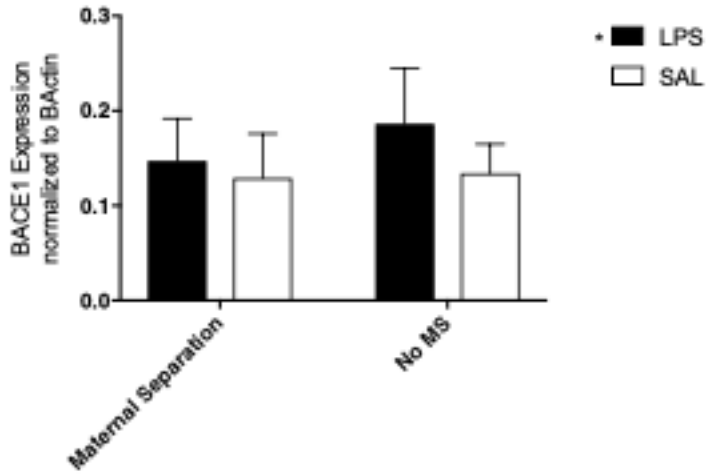

Figure 8. Maternal separation did not increase BACE1 levels in females. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more hippocampal BACE1 than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

Discussion

These experiments aimed to determine whether maternal separation increases hippocampal amyloid beta and results in cognitive deficits in C57BL/6J mice. Maternal separation has been shown to cause a long-lasting disruption in HPA axis function (Clarke, 1993; Slotten et al., 2006; Roque et al., 2014), memory deficits, decreased hippocampal levels of BDNF (Pinheiro et al., 2014) as well as increased BACE1 expression and increases in

hippocampal $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels (Martisova, Aisa, Guerenu, & Ramirez, 2013). The present studies sought to determine whether maternal separation exacerbated LPS-induced hippocampal Aβ accumulation and cognitive deficits. The hypotheses were partially supported. Contextual fear conditioning data revealed that non-maternally separated males froze significantly more than did males that were maternally separated, indicating that maternal separation impaired the animals' ability to associate the context shock pairing. Additionally, maternally separated males had increased hippocampal BACE1 expression. This could indicate sex specificity in the response to the stress. Animals did not differ significantly from non-maternally separated animals in hippocampal Aβ level.

As hypothesized, maternally separated males displayed a cognitive deficit in CFC after 7, once-daily injections. This is consistent with previous research showing maternal separation leads to cognitive deficits (Pinheiro et al., 2014; Hui et al., 2017; Lesuis et al., 2018a) in various tasks including the Morris water maze and novel object recognition tasks (Aisa et al., 2007). In BALB/cJ mice, maternal separation led to deficits in auditory cued fear conditioning in both sexes, novel object recognition in females, and spatial and reversal learning in males (Wang, Jiao, & Dulawa, 2011). However, this effect is not uniform, as Mehta & Schmauss (2011) found no cogntive deficit in a spatial working memory task in C57BL/6 mice following maternal separation. There was not the hypothesized LPS treatment main effect in males, but this appeared to be due to the decrease in freezing in the maternally separated saline animals lowering the saline group mean. It is possible that adding more animals in a future study would pull through an interaction effect.

Contrary to the hypothesis, there were no significant effects in contextual fear conditioning in females. The groups did appear to be trending in the right direction, and the study

may have been underpowered as the n was slightly lower than in males. However, this failure to find an effect is consistent with research by Loi et al. (2017) demonstrating no effect of maternal deprivation on females in the contextual memory tasks novel object recognition and location. Other researchers have, alternatively, found significant cognitive impairment in females (Sun et al., 2014). Age at testing also appears to play a role, as Sun et al. (2014) found the effect in females when testing on post-natal day 90, earlier than the age at testing in the present experiment. Additionally, research has shown there is some sex specificity in the effects of maternal separation on later cognition. In one study, maternal separation affected the HPA axis more significantly in female rats, whereas males displayed a more significant cognitive deficit in T-maze acquisition (Slotten et al., 2006). It is possible that cognition in males is more impacted by maternal separation under the paradigm of maternal separation utilized in this study. Further research is needed to explore the parameters underlying the effects of early life stress on cognition in adulthood.

It was also hypothesized that maternal separation and LPS would impact AD related pathology. The hypotheses were partially supported. Consistent with previous research from our lab, males and females given 7 days of LPS had increased hippocampal Aβ as compared with saline-treated animals (Kahn et al., 2012). However, maternally separated animals did not differ significantly from non-maternally separated animals in hippocampal Aβ level and there was not an interaction between LPS and maternal separation, indicating that there may be a ceiling effect, where 7 days of LPS administration produces more Aβ than can be altered by maternal separation. In addition, maternally separated males had a marginally significant increase in BACE1 expression, while females did not. This is consistent with the fear conditioning data, and indicates there may be a marginal increase in amylogenic processing of APP in males. This is

somewhat consistent with the literature, where maternal separation has increased BACE1 expression though it did increase hippocampal $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels (Martisova et al., 2013). A potential explanation for the sex specificity is a trend in the recent literature indicating females may be more resilient to the hippocampal effects of childhood maltreatment than males (Loi et al., 2017).

These results demonstrate an interaction between an early life stressor, maternal separation, and Alzheimer's disease like pathology later in life. Though LPS-treated maternally separated animals did not differ from non-maternally separated LPS-treated animals in hippocampal Aβ level, contextual fear conditioning data revealed that maternally separated males displayed impaired ability to associate the context shock pairing. Additionally, maternally separated males had increased hippocampal BACE1 expression, the main β-secretase. It is possible that there was no significant increase in hippocampal Aβ as a result of the LPS injections because 7, once-daily injections leads to too much Aβ for maternal separation to exacerbate, consistent with a potential ceiling effect. The next study was conducted to investigate this possibility.

CHAPTER 3: THE EFFECTS OF MATERNAL SEPARATION AND 3 LPS INJECTIONS

Abbreviated Introduction

Our laboratory has previously shown that repeated administrations of LPS induce inflammation, cause cognitive deficits, and increase Aβ levels in the central nervous system (Kahn et al., 2012). This research from our lab demonstrated there were significant elevations in Aβ levels after 7, once-daily LPS injections but not yet after just 3 LPS injections. Since maternal separation can result in increased proinflammatory cytokine production and cognitive deficits in non-transgenic animals (Pinheiro et al., 2014), and increase AD-like pathology in AD transgenic mice (Hui et al., 2017), the potential interaction between MS and adulthood LPS administration necessitates investigation. Experiment 1 demonstrated there is increased BACE1 expression and a cognitive deficit in males following 7, once-daily injections. However, there was not a significant increase in hippocampal Aβ as a result of the LPS injections, potentially because 7, once-daily injections lead to too much Aβ for maternal separation to exacerbate, and that there is some sort of ceiling effect. The purpose of the present study was to determine whether maternal separation would interact with LPS administration and increase cytokine or Aβ levels after only 3, once-LPS injections. The previous study also investigated cognitive impairments following maternal separation and LPS administration, and this study sought to extend those findings.

The purpose of the present research is to extend the previous research and investigate these effects at an additional time point, as well as explore factors that may play a mechanistic role in these effects. For this reason, it was further hypothesized that animals with a cognitive deficit would also display decreased expression of proBDNF, the precursor to BDNF. Additionally, it was hypothesized maternally separated animals displaying increased Aβ would

have coinciding increases in peripheral levels of cytokines IL-1β, IL-10, and TNF-α. Because AD is linked to the inflammatory response, a factor capable of exacerbating inflammation could indirectly alter disease pathogenesis. Since proinflammatory cytokines are induced by LPS injections, and contribute to the LPS-induced accumulation of hippocampal Aβ, it was hypothesized that animals with increased hippocampal Aβ would also display increases in the proinflammatory cytokines IL-1β and TNF-α. IL-10 is more traditionally classified as an anti-inflammatory cytokine, but has also been shown to increase following LPS administration, potentially as a compensatory mechanism (Chanteux et al., 2007). It was therefore hypothesized maternal separation may also further increase compensatory production of IL-10 following LPS.

The present experiment aimed to test the hypothesis that an early life stressor, maternal separation, would significantly exacerbate LPS-induced soluble Aβ production and cognitive deficits in C57BL/6J mice in comparison to non-maternally separated animals. More specifically, it was hypothesized that there would be a significant increase in Aβ in maternally separated, LPS-treated animals over non-maternally separated and saline-treated controls. Further, it was hypothesized that maternally separated LPS-treated mice would perform worse in contextual fear conditioning than non-maternally separated or saline-treated animals. Experiment 1 demonstrated there is increased BACE1 expression and a cognitive deficit in males following 7, once-daily LPS injections. The purpose of the present research is to extend these findings and investigate these effects at an additional time point, as well as exploring factors that play a mechanistic role in these effects. For this reason, it was further hypothesized that animals with a cognitive deficit would also display decreased proBDNF expression. Additionally, it was hypothesized maternally separated animals displaying increased Aβ would have coinciding increases in peripheral levels of cytokines IL-1B, IL-10, and TNF-α.

Methods

Unless otherwise stated, all subjects, behavioral paradigms, and tissue collection and analysis protocols were identical to those described above.

Treatment conditions

On post-natal day 2 (PND 2; Solas et al., 2010), pups from all litters were sexed, mixed, and randomly assigned to each dam (Roque at al., 2014). Each dam and the corresponding litter were randomly assigned to experimental conditions: Maternal Separation (MS) or Control (C). Maternally separated pups were separated from the dam for 3h daily from PND 2–21, and control pups remained with the dam till weaning at PND 23 (Solas et al., 2010) Maternally separated animals were transferred with nesting material and were monitored in an incubator to maintain constant temperature of 31°C. At weaning all animals were grouped by sex into groups of 3 or 4 and were allowed to age normally until adulthood. At 4–6 months of age, males were randomly assigned into the following treatment conditions: MS + LPS, MS + Saline, C + LPS, or C + Saline. Following group assignment, animals were given 3, once-daily injections of either 250μg/kg of LPS (*Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) or Saline as a control. LPS was administered in a weight-dependent manner to ensure the dose met a concentration of 250 μg/kg. Twenty-four hours after the final injection, animals underwent a contextual fear-conditioning paradigm and then blood and tissue were collected.

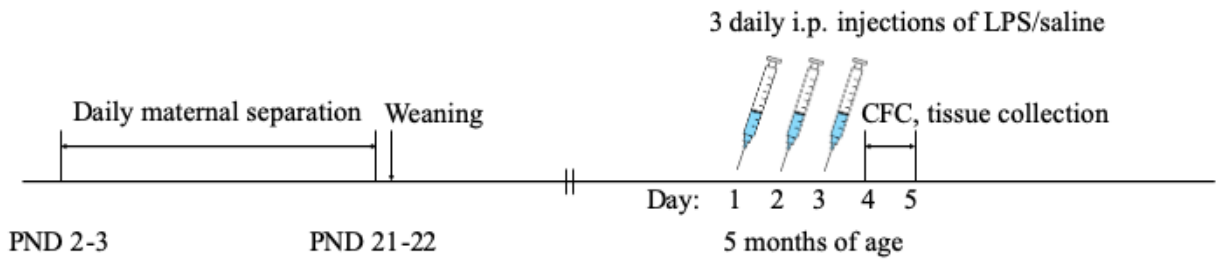

Figure 9. Experimental timeline: Experiment 2. MS animals were maternally separated from PND2-2 while non-MS animals were undisturbed, and then both groups aged until adulthood and then were administered 3 LPS injections prior to CFC and tissue collection.

Western blotting

Samples were diluted in sample buffer to a working concentration of 1 g/L and then denatured via boiling at 100◦ C for 5 m in the presence of β-mercaptoethanol (Sigma, St. Louis, MO) to reduce disulfide bonds. Proteins were separated by size with Mini-Protean® TGXTM precast gradient gels (4–15%; BioRad, Hercules, CA). 200 V was applied across the gel for 40 minutes, the gel was removed and equilibrated in Towbin transfer buffer. Then proteins were transferred onto a polyvinylidene fluoride (PVDF) (Millipore, Billerica, MA) membrane at 0.15 amps per gel for 40 minutes with a semi-dry transfer unit (BioRad). After transfer, membranes were blocked using 5% bovine serum albumin in Tris buffered saline with 0.3% Tween® 20 (TBST) for 2 hours. Primary antibodies (proBDNF 1:400, β-actin 1:500) were diluted in TBST, applied to the membrane and incubated overnight at 4◦ C. The next day, membranes were washed in TBST and then peroxidase-conjugated AffiniPure secondary antibodies were applied (Jackson ImmunoResearch Laboritories, Inc., West Grove, PA) for 2h at room temperature. Membranes were washed and then coated with the detection reagent, SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Wiltham, MA). Chemiluminescence was imaged using a Syngene G:Box (Syngene, Fredrick, MD), and densitometry analysis was

performed using GeneTools software (Syngene, Fredrick, MD). β-actin was used as the loading control.

Multiplexing

Whole blood samples were collected and then stored on ice for 15 minutes, removed and allowed to clot for 30 minutes, and then centrifuged at 2000 xg for 10 minutes. The serum was collected and stored at -80◦ C until the time of the assay. A multiplex for cytokines IL-1β, IL-10, and TNF-α was conducted according to manufacturer's instructions (Meso Scale Discovery, Rockville, MD). The plate is read on a high-sensitivity MESO^{TM} QuickPlex SQ 120 machine, which utilizes electrochemiluminescence.

4.0 Results

Experiment 2A: Effects of Maternal Separation and 3 LPS Injections on Cognition

Maternal separation leads to impaired freezing in contextual fear conditioning in males

A two-way analysis of variance (ANOVA) was used to determine whether maternal separation impacted cognition in a contextual fear conditioning paradigm. Analyses revealed a main effect of Treatment (LPS or Saline); $F(1, 29)= 4.920$, $p = .035$) such that LPS-treated animals froze significantly less in CFC than saline-treated animals. There was a marginally significant Condition x Genotype interaction ($F(1, 29)= 4.168$, $p = .050$), where MS saline-treated animals froze less than non-MS saline-treated animals. There was no main effect of Condition (MS or no MS; $F(1, 29)= .010$, *NS*; Figure 10). Overall, maternal separation and LPS led to impaired freezing in contextual fear conditioning.

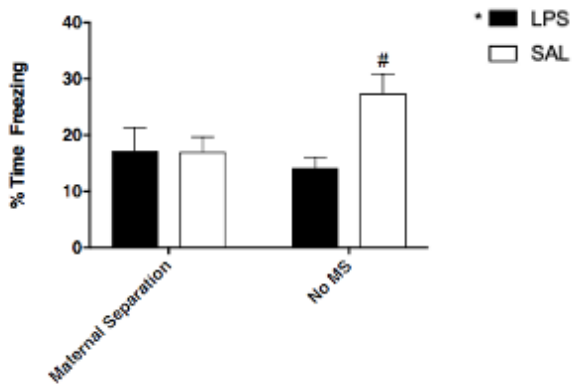

Figure 10. Maternal separation leads to impaired cognition in males. A 2x2 ANOVA revealed a significant main effect of treatment and a marginally significant interaction effect where the LPS-treated animals and the saline maternally separated animals froze less than the saline non-maternally separated control animals. Bars represent mean ± SEM. Significance differences (p<0.05) are designated by *. Marginally significance differences (p=0.05) are designated by #.

Maternal separation leads to impaired freezing in contextual fear conditioning in females

A two-way analysis of variance (ANOVA) was used to determine whether maternal separation impacted cognition in a contextual fear conditioning paradigm. Analyses revealed a main effect of Condition (MS or no MS; $F(1, 60)= 5.678$, $p = .020$) such that MS animals froze significantly less in CFC than non-MS animals. There was not a significant main effect of Treatment (LPS or Saline; $F(1, 60)= .001$, *NS*) or a significant Condition x Genotype interaction ($F(1, 60)= .089$, *NS*; Figure 11). Overall, maternal separation led to impaired freezing in contextual fear conditioning.

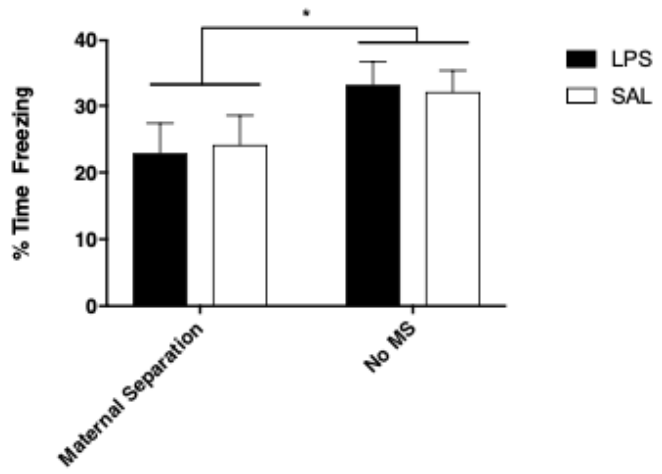

Figure 11. Maternal separation leads to impaired cognition in females. A 2x2 ANOVA revealed a main effect of condition, such that maternally separated animals froze significantly less than control animals regardless of treatment. Bars represent mean ± SEM. Significance differences (p<0.05) are designated by *.

Experiment 2B: Effects of Maternal Separation and 3 LPS Injections on Aβ

3 LPS injections significantly increase hippocampal Aβ levels in males

A 2x2 ANOVA was used to determine whether maternal separation impacted hippocampal Aβ levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 29)$ 9.928, $p = .004$) such that LPS-treated animals had significantly more hippocampal Aβ than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 29) = .077$, *NS*) or a significant Condition x Treatment interaction ($F(1, 29) = .200$, *NS*); Figure 12). Overall, LPS, but not maternal separation, significantly increase hippocampal Aβ levels.

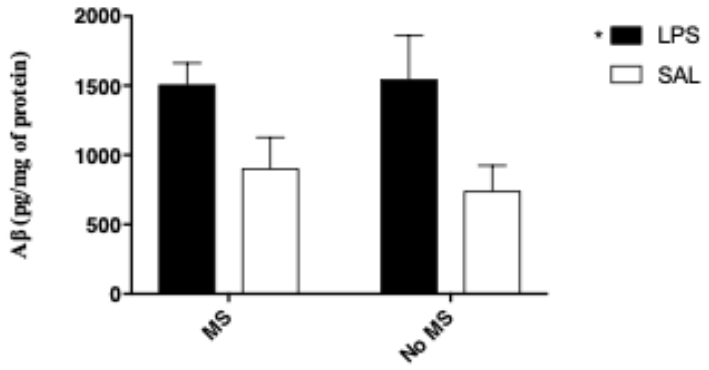

Figure 12. Maternal separation did not increase soluble Aβ levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more hippocampal Aβ than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

3 LPS injections and maternal separation significantly increase hippocampal Aβ levels in females

A 2x2 ANOVA was used to determine whether maternal separation impacted hippocampal Aβ levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 22)$ 9.962, p = .005) such that LPS-treated animals had significantly more hippocampal Aβ than saline-treated animals. There was also a marginally significant Condition x Treatment interaction ($F(1, 22)$= 4.197, p = .053) such that LPS-treated MS animals had the most Aβ. There was not a significant main effect of Condition (MS or no MS; $F(1, 22)$= .659, *NS*; Figure 13). Overall, maternal separation and 3, once-daily LPS injections together significantly increased hippocampal Aβ levels.

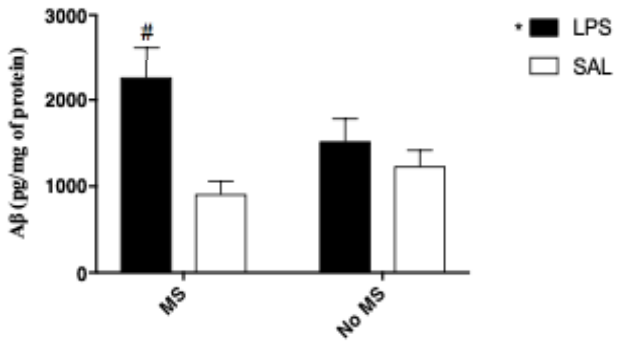

Figure 13. Maternal separation increased soluble Aβ levels in females. A 2x2 ANOVA revealed a main effect of treatment and a marginally significant interaction effect such that LPS MS animals had significantly more hippocampal Aβ than saline or non-MS animals. Significance differences (p<0.05) are designated by *. Marginally significance differences (p=0.053) are designated by #.

Experiment 2C: Effects of Maternal Separation and 3 LPS Injections on proBDNF

Maternal separation significantly decreases hippocampal proBDNF levels in males

A 2x2 ANOVA was used to determine whether maternal separation impacted hippocampal BACE1 levels. Because the Levene's test of equality of error variances revealed significant heterogeneity of variance, F= 4.613, an ANOVA was conducted on the natural log of raw scores. Analyses revealed a main effect of Condition (MS or no MS; F(1, 34) 8.844, p = .005) such that MS animals had significantly decreased hippocampal proBDNF as compared to no MS animals. There was not a significant main effect of Treatment (LPS or saline; F(1, 34)= .018, *NS*) or a significant Condition x Treatment interaction (F(1, 34)= 2.607, *NS*); Figure 14). Overall, maternal separation significantly decreased hippocampal proBDNF levels.

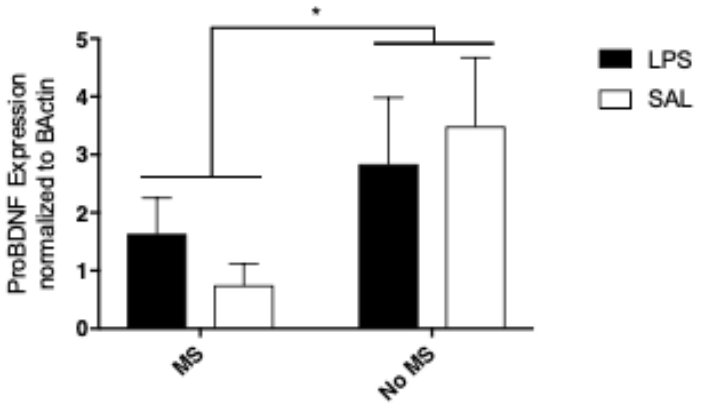

Figure 14. Maternal separation decreased proBDNF levels in males. A 2x2 ANOVA revealed a main effect of condition such that MS animals had significantly less hippocampal proBDNF than no MS animals regardless of treatment. Significance differences (p<0.05) are designated by *.

Maternal separation significantly decreases hippocampal proBDNF levels in females

A 2x2 ANOVA was used to determine whether maternal separation impacted hippocampal BACE1 levels. Analyses revealed a main effect of Condition (MS or no MS; F(1, 19) 8.437, p = .009) such that MS animals had significantly decreased hippocampal proBDNF as compared to no MS animals. There was not a significant main effect of Treatment (LPS or saline; F(1, 19)= .182, *NS*) or a significant Condition x Treatment interaction (F(1, 19)= .225, *NS*); Figure 15). Overall, maternal separation significantly decreased hippocampal proBDNF levels.

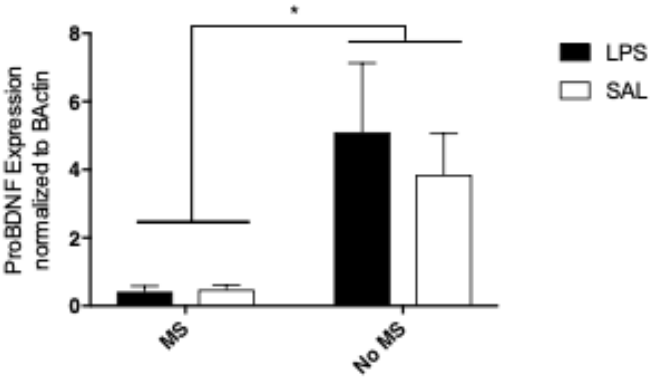

Figure 15. Maternal separation decreased proBDNF levels in females. A 2x2 ANOVA revealed a main effect of condition such that MS animals had significantly less hippocampal proBDNF than no MS animals regardless of treatment. Significance differences (p<0.05) are designated by *.

Experiment 2D: Effects of Maternal Separation and 3 LPS Injections on IL-1β, TNF-α and IL-10 in males

3 LPS injections significantly increase serum IL-1β levels in males

A 2x2 ANOVA was used to determine whether maternal separation impacted serum IL-1β levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 23)$ 19.826, $p <$ .001) such that LPS-treated animals had significantly more serum IL-1β than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 23)= .103$, *NS*) or a significant Condition x Treatment interaction ($F(1, 23)= .439$, *NS*); Figure 16). Overall, LPS, but not maternal separation, significantly increased serum IL-1β levels.

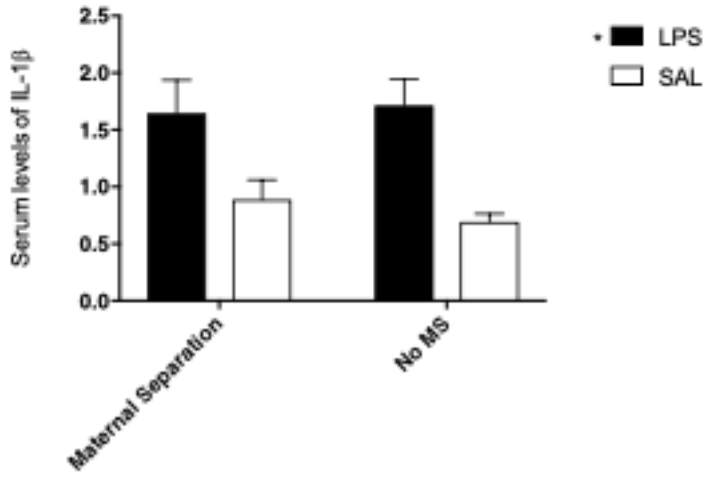

Figure 16. Maternal separation did not significantly impact IL-1β levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum IL-1β than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

3 LPS injections significantly increase serum TNF-α levels in males

A 2x2 ANOVA was used to determine whether maternal separation impacted serum TNF-α levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 23)$ 15.117, p = .001) such that LPS-treated animals had significantly more serum TNF-α than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 23)$= .059, *NS*) or a significant Condition x Treatment interaction ($F(1, 23)$= .319, *NS*); Figure 17). Overall, LPS, but not maternal separation, significantly increased serum TNF-α levels.

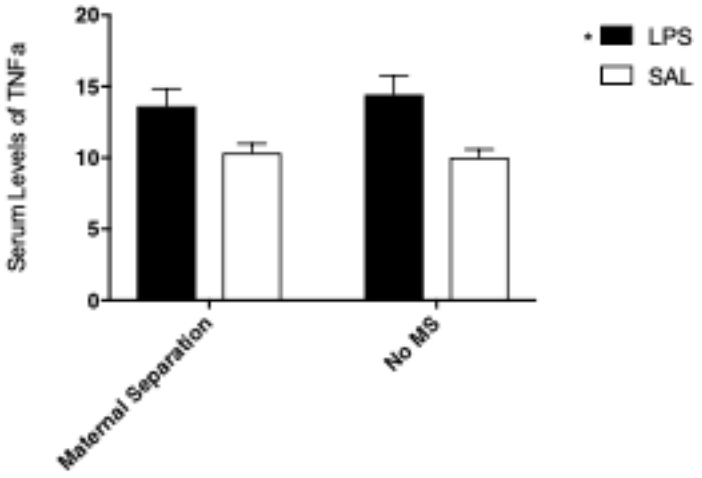

Figure 17. Maternal separation did not significantly impact serum TNF-α levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum TNF-α than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

3 LPS injections significantly increase serum IL-10 levels in males

A 2x2 ANOVA was used to determine whether maternal separation impacted serum IL-10 levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 23)$ 222.609, p < .001) such that LPS-treated animals had significantly more serum IL-10 than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 23)$= .577, *NS*)

or a significant Condition x Treatment interaction ($F(1, 23)= .958$, *NS*); Figure 18). Overall, LPS, but not maternal separation, significantly increased serum IL-10 levels.

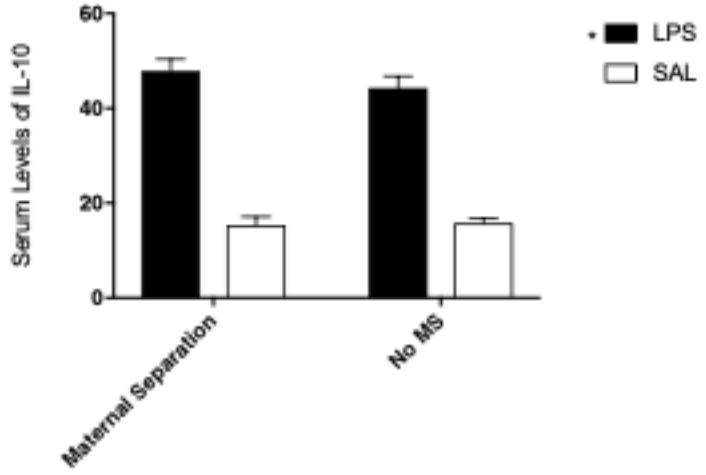

Figure 18. Maternal separation did not significantly impact serum IL-10 levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum IL-10 than saline animals regardless of condition. Significance differences ($p<0.05$) are designated by *.

Discussion

The purpose of the present research was to extend the findings of Experiment 1 and investigate significant effects at an additional time point, as well as explore factors that may play a mechanistic role in these effects. These experiments aimed to determine whether maternal separation increases hippocampal amyloid beta and results in cognitive deficits in C57BL/6J mice. Maternal separation has been shown to cause a long-lasting disruption in HPA axis function (Clarke, 1993; Slotten et al., 2006; Roque et al., 2014), memory deficits, decreased hippocampal levels of BDNF (Pinheiro et al., 2014) as well as increased BACE1 expression as well as increases in hippocampal $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels (Martisova, Aisa, Guerenu, & Ramirez, 2013). The present studies sought to test the effects of maternal separation and 3, once-daily LPS injections on hippocampal Aβ and cognition. The hypotheses were partially supported.

Contextual fear conditioning data revealed that non-maternally separated animals froze significantly more than did animals that were maternally separated, indicating that maternal separation impaired the animals' ability to associate the context shock pairing. Additionally, maternally separated females had increased hippocampal Aβ after 3, once-daily LPS injections. Males did not differ significantly from non-maternally separated animals in hippocampal Aβ level. This could indicate sex specificity in response to the stress. Males and females that were maternally separated demonstrated decreases in hippocampal proBDNF expression, but not increases in the cytokines TNF-α, IL-10, or IL-1β.

As hypothesized, maternally separated males and females displayed a cognitive deficit in CFC after 3, once-daily LPS injections. This is consistent with previous research showing maternal separation leads to cognitive deficits (Pinheiro et al., 2014; Hui et al., 2017; Lesuis et al., 2018a) in the Morris water maze and novel object recognition tasks (Aisa et al., 2007) as well as auditory cued fear conditioning (Wang et al., 2011). However, this effect is not uniform, as Mehta & Schmauss (2011) found no cogntive deficit in a spatial working memory task in C57BL/6 mice following maternal separation. A potential mechanism behind impared cognition following stress is decreased BDNF expression. As hypothesized, maternally separated males and females demonstrated decreased hippocampal proBDNF expression. This is consistent with the literature showing maternal separation decreases levels of BDNF in adult animals (Pinheiro et al., 2014).

It was also hypothesized that maternal separation and LPS would impact AD-like pathological markers. The hypotheses were partially supported. Maternally separated females demonstrated marginally significantly increased hippocampal Aβ after 3, once-daily LPS injections. This is consistent with the literature that showing early life stress can impact AD

related pathology. Maternal separation and bedding restriction cause increased BACE1 expression (Martisova et al., 2013; Lesuis et al., 2018a) as well as increases in hippocampal $A\beta_{1-40}$ and $A\beta_{1-42}$ levels (Martisova et al., 2013; Lesuis et al., 2018a) and, in transgenic animals, Aβ plaques (Hoeijmakers et al. 2017; Hui et al., 2017). However, males did not differ significantly from non-maternally separated animals in hippocampal Aβ level. This could indicate sex specificity in response to the stress and is consistent with the research of Slotten et al. (2006), who found that maternal separation affected the HPA axis more significantly in female rats, while males displayed more significant cognitive deficits. The somewhat ambiguous nature of the effects of maternal separation necessitates further research to determine how early life stress can alter cognition and the development of AD pathology later in life.

It was also hypothesized that maternal separation would lead to an increase in proinflammatory cytokines peripherally following LPS injections. LPS has been shown to increase peripheral cytokines, which potentially lead to the LPS induced accumulation of hippocampal Aβ (Kahn et al., 2012). It was hypothesized that animals with increased hippocampal Aβ would also display increases in the proinflammatory cytokines IL-1β and TNF-α. It was further hypothesized maternal separation may further increase the compensatory production of IL-10. As hypothesized, LPS increased the serum levels of IL-1β, TNF-α, and IL-10. However, maternal separation did not further increase these peripheral cytokine levels. This was consistent with previous research showing that the cytokines IL-1β and TNF-α were not exacerbated by maternal separation following inflammation (Genty, Nomigni, Anton & Hanesch, 2018). However, Lesuis et al., (2018b) indicates programming of the HPA axis and priming of the neuroinflammatory response as potential mechanisms for early life stress modulating AD neuropathology and cognition. Further, Pinheiro et al. (2014) demonstrated that maternal

separation increased the levels of IL-10 and TNF-α in the hippocampus. However, it may be that peripheral cytokines are not affected, while central cytokines are increased. Moreover, the duration of the stressor may play a role in the variability, as well as the timing of testing.

These results demonstrate an interaction between an early life stressor, maternal separation, and Alzheimer's disease-like pathology later in life. LPS-treated maternally separated females had more hippocampal Aβ than non-maternally separated LPS-treated animals and contextual fear conditioning data revealed that maternally separated animals displayed impaired ability to associate the context-shock pairing. Additionally, maternally separated animals had decreased hippocampal proBDNF expression. It is possible that there was no significant increase in cytokine levels as a result of the LPS injections because the difference in cytokines was greatest on a different day of the LPS injection schedule and the magnitude of the cytokine response decreased over the days, as previous research from our lab has demonstrated a robust increase in cytokines 4 hours following the first LPS injection that is significantly decreased by day 7. Three injections may be too late to see differences in MS-induced increases in cytokine levels in response to LPS, as the difference in cytokines was greatest on day 1 of LPS administration. The next study was conducted to investigate this possibility.

CHAPTER 4: THE EFFECTS OF MATERNAL SEPARATION AND 1 LPS INJECTION

Abbreviated Introduction

Our laboratory has previously shown that repeated administrations of LPS induce inflammation (Kahn et al., 2012). Since research has shown maternal separation can result in increased proinflammatory cytokine production in non-transgenic animals (Pinheiro et al., 2014), the present studies have investigated potential interaction between MS and adulthood LPS administration. The purpose of the present study was to determine if maternal separation and LPS administration would increase cytokine levels after 1 LPS injection. Our laboratory has shown that serum IL-1β levels were most elevated after 1 LPS injection, as compared with following 4 or 7 injections (Kahn et al., 2012). For this reason, it was hypothesized the greatest difference in cytokines between maternally separated and non-maternally separated animals would be following the first LPS injection and the present study sought to investigate this hypothesis. Blood was not collected after the first LPS injection in the previous two experiments, but instead at the time of tissue collection, so as not to confound any of the other data collection. In the present experiment, animals were euthanized following the first LPS injection.

In addition to its impacts on AD, early life stress has also been established as a risk factor for the development of mood and anxiety disorders in humans (Heim et al., 2010). Research demonstrates that maternal separation can impact anxiety like behavior. Romeo et al. (2003) found that maternally separated C57BL/6 males displayed higher levels of anxiety behaviors while maternally separated females displayed reduced anxiety in open field testing. However, the literature also supports differing effects, as maternally separated rats have displayed hyperlocomotion and more exploration (Wongwitdecha, Yoopan, & Srisomboonlert, 2008), while other researchers have found maternally separated females spent less time in the center

during open field testing (Tsuda & Ogawa, 2012). Researchers have shown that the effects of maternal separation on later open field behavior may be timing-dependent, as maternal separation from post natal day 2–15 led to decreased time spent in center in open field testing, but not in animals maternally separated on post natal day 7–20 (Roque et al., 2014). Anxiety like behaviors were not quantified in the previous studies so as not to confound the contextual fear conditioning data. It was hypothesized that maternal separation would alter the open field behavior of the maternally separated males and female mice as compared to the non-maternally separated animals.

The proposed study aimed to explore the interaction between maternal separation, LPS, and cytokine levels, in addition to investigating the effects of maternal separation on anxiety like behaviors. It was hypothesized that maternal separation would significant increase levels of serum IL-1β, TNF-α, and IL-10 following 1 LPS injection as compared with saline, and non-maternally separated control animals. It was also hypothesized that maternal separation would alter time spent exploring the center of the open field chamber as compared with non-maternally separated animals in open field behavioral testing.

Methods

Unless otherwise stated, all subjects, behavioral paradigms, and tissue collection and analysis protocols were identical to those described above.

Treatment conditions

On post-natal day 2 (PND 2; Solas et al., 2010), pups from all litters were sexed, mixed, and randomly assigned to each dam (Roque at al., 2014). Each dam and the corresponding litter were randomly assigned to experimental conditions: Maternal Separation (MS) or Control (C). Maternally separated pups were separated from the dam for 3h daily from PND 2-21, and control

pups remained with the dam till weaning at PND 23 (Solas et al., 2010) Maternally separated animals were transferred with nesting material and were monitored in an incubator to maintain constant temperature of 31°C. At weaning all animals were grouped by sex into groups of 3 or 4 and were allowed to age normally until adulthood. At 4–6 months of age, animals underwent open field behavioral testing. A week later, animals were randomly assigned into the following treatment conditions: MS + LPS, MS + Saline, C + LPS, or C + Saline. Following group assignment, animals were given 1 daily injection of either 250µg/kg of LPS (*Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) or Saline as a control. LPS was administered in a weight-dependent manner to ensure the dose met a concentration of 250 µg/kg. Four hours after the injection blood and tissue were collected.

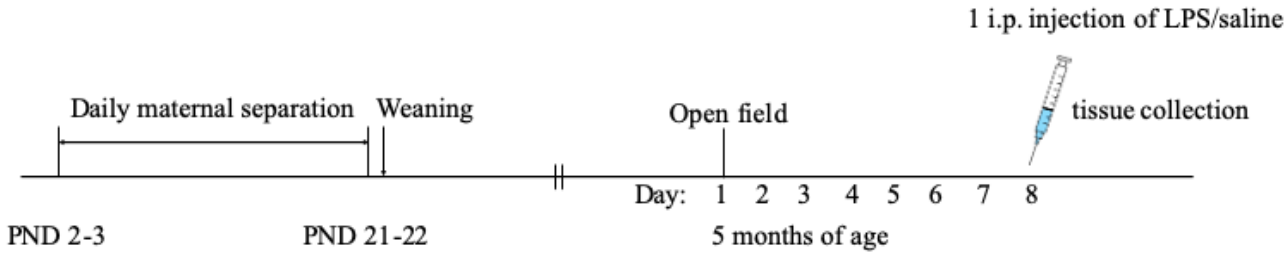

Figure 19. Experimental timeline: Experiment 3. MS animals were maternally separated from PND2-2 while non-MS females were undisturbed, and then both groups aged until adulthood and then underwent open field behavioral testing. One week later animals were administered 1 LPS injection and tissue collection was 4 hours later.

Behavioral Paradigm

At approximately 5 months of age, male and female animals underwent open field behavioral testing prior to administration of LPS or saline. The open field task examines exploratory and anxiety-like behaviors. Animals were removed from their home cages and placed in the center of the open field chamber, where they were allowed uninterrupted movement during the 10 minute session. Following testing, animals were returned to their home cages. Testing was conducted in four open field maze chambers (27 x 27 cm) while video tracking software (Med Associates

Incorporated, St. Albans, VT) quantified the animals' locomotor activity. Zones were predetermined (zone 1: center of the chamber, zone 2: the remainder of the area in chamber) in order to quantify the amount of time spent in the center verses the periphery. The three-dependent variables measured were the total ambulatory distance traveled (cm), the duration of time spent in the center zone (s), and the average speed (cm/s).

Results

Experiment 3A: Effects of Maternal Separation on Open Field Behavior in Males

Maternal Separation leads to decreased time spent in the center in males

A Student's t-test was used to examine differences across Condition (Maternal Separation or No Maternal Separation) in time spent in the center of the open field chamber. Analyses revealed significant differences in time spent in the center between maternally separated and non-maternally separated males ($t(1, 41)= -2.082$, $p = .044$); Figure 20). Overall, maternally separated males spent less time in the center than did non-maternally separated controls.

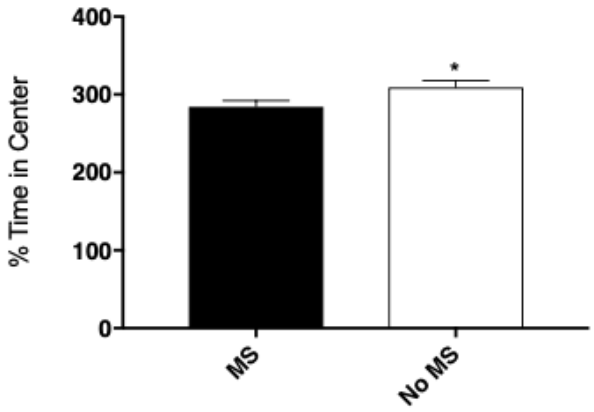

Figure 20. Maternal separation leads to less time spent in the center in male mice. Student's t-tests revealed significant differences in time spent in the center between maternally separated and non-maternally separated animals. Bars represent mean ± SEM. Significance differences ($p<0.05$) are designated by *.

Maternal Separation leads to increased distance travelled in males

A Student's t-test was used to examine differences across Condition (Maternal Separation or No Maternal Separation) in distance travelled in the open field chamber. Analyses revealed significant differences in time spent in the center between maternally separated and non-maternally separated males ($t(1, 41)= 3.128$, $p = .003$); Figure 21). Overall, maternally separated males travelled more distance than did non-maternally separated controls.

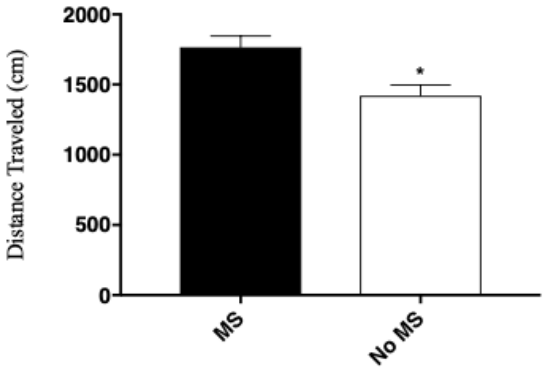

Figure 21. Maternal separation leads to increased distance travelled in male mice. Student's t-tests revealed significant differences in distance travelled between maternally separated and non-maternally separated animals. Bars represent mean ± SEM. Significance differences ($p<0.05$) are designated by *.

Maternal Separation does not alter average speed in males

A Student's t-test was used to examine differences across Condition (Maternal Separation or No Maternal Separation) in average speed in the open field chamber. Analyses revealed no significant differences in average speed between maternally separated and non-maternally separated males ($t(1, 41)= -.527$, *NS*); Figure 22). Overall, maternally separated males travelled at the same speed as non-maternally separated controls.

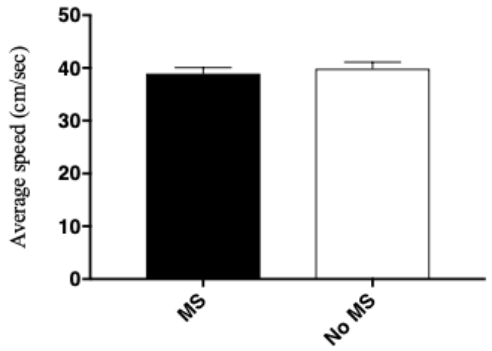

Figure 22. Maternal separation does not alter average speed in male mice. Student's t-tests revealed no significant differences in average speed between maternally separated and non-maternally separated animals. Bars represent mean ± SEM. Significance differences (p<0.05) are designated by *.

Experiment 3B: Effects of Maternal Separation on Open Field Behavior in Females

Maternal Separation leads to increased time spent in the center in females

A Student's t-test was used to examine differences across Condition (Maternal Separation or No Maternal Separation) in time spent in the center of the open field chamber. Analyses revealed significant differences in time spent in the center between maternally separated and non-maternally separated females (t(1, 40)= 2.871, p = .007); Figure 23). Overall, maternally separated females spent more time in the center than did non-maternally separated controls.

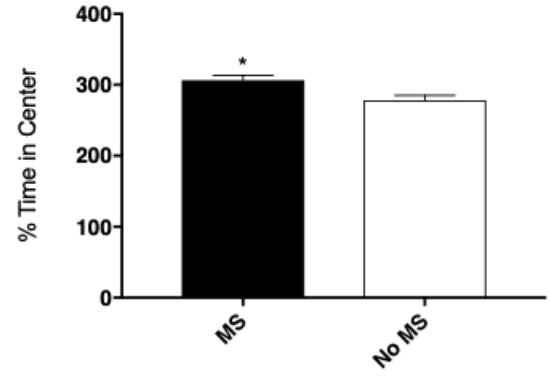

Figure 23. Maternal separation leads to more time spent in the center in female mice. Student's t-tests revealed significant differences in time spent in the center between maternally separated and non-maternally separated animals. Bars represent mean ± SEM. Significance differences (p<0.05) are designated by *.

Maternal Separation does not alter distance travelled in females

A Student's t-test was used to examine differences across Condition (Maternal Separation or No Maternal Separation) in distance travelled in the open field chamber. Analyses revealed no significant differences in distance travelled between maternally separated and non-maternally separated males ($t(1, 40)=.274$, *NS*); Figure 24). Overall, maternally separated females travelled the same distance as non-maternally separated controls.

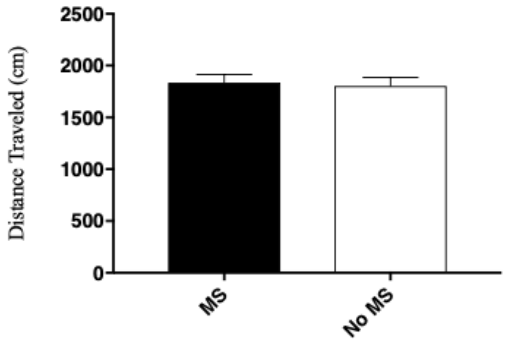

Figure 24. Maternal separation does not alter distance travelled in female mice. Student's t-tests revealed no significant differences in distance travelled between maternally separated and non-maternally separated animals. Bars represent mean ± SEM. Significance differences ($p<0.05$) are designated by *.

Maternal Separation does not alter average speed in females

A Student's t-test was used to examine differences across Condition (Maternal Separation or No Maternal Separation) in average speed in the open field chamber. Analyses revealed no significant differences in average speed between maternally separated and non-maternally separated females ($t(1, 40)= -.942$, *NS*); Figure 25). Overall, maternally separated females travelled the same distance as non-maternally separated controls.

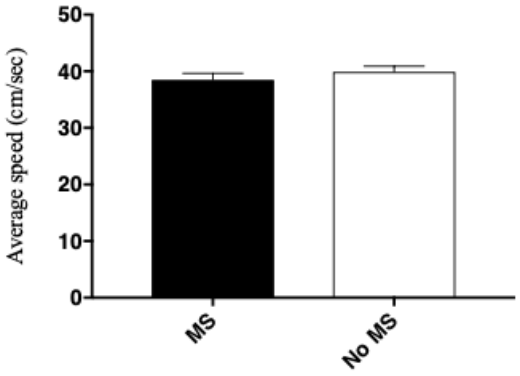

Figure 25. Maternal separation does not alter average speed in female mice. Student's t-tests revealed no significant differences in average speed between maternally separated and non-maternally separated animals. Bars represent mean ± SEM. Significance differences (p<0.05) are designated by *.

Experiment 3C: Effects of Maternal Separation and 1 LPS Injection on IL-1β

One LPS injection significantly increases serum IL-1β levels in males

A 2x2 ANOVA was used to determine if maternal separation impacted serum IL-1β levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 36)= 49.806$, $p < .001$) such that LPS-treated animals had significantly more serum IL-1β than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 36)= .006$, *NS*) or a significant Condition x Treatment interaction ($F(1, 36)= .007$, *NS*); Figure 26). Overall, LPS, but not maternal separation, significantly increased serum IL-1β levels.

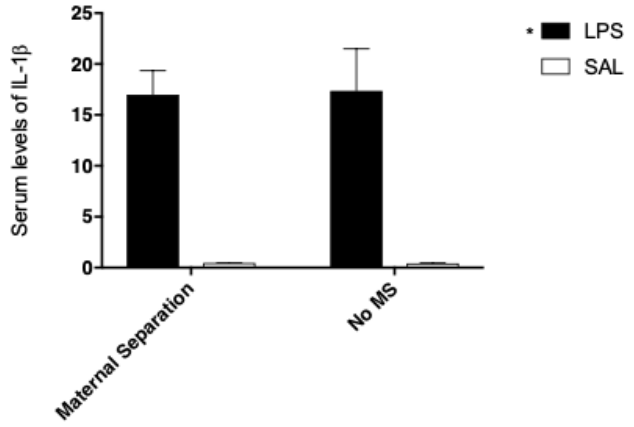

Figure 26. Maternal separation did not significantly impact IL-1β levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum IL-1β than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

One LPS injection significantly increases serum IL-1β levels in females

A 2x2 ANOVA was used to determine if maternal separation impacted serum IL-1β levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 36)= 44.647$, $p < .001$) such that LPS-treated animals had significantly more serum IL-1β than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 36)= .016$, *NS*) or a significant Condition x Treatment interaction ($F(1, 36)= .022$, *NS*); Figure 27). Overall, LPS, but not maternal separation, significantly increased serum IL-1β levels.

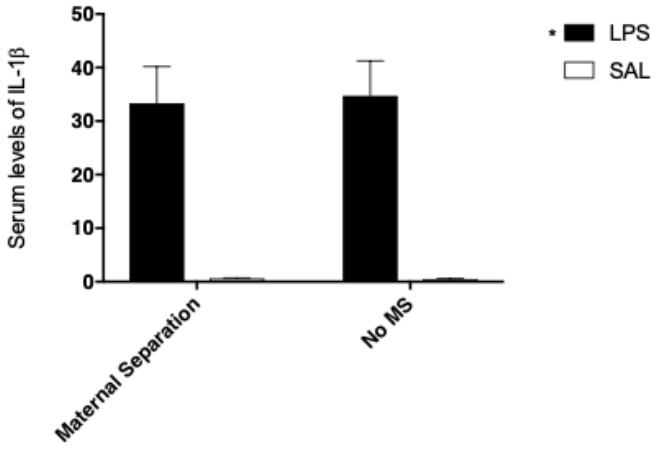

Figure 27. Maternal separation did not significantly impact IL-1β levels in females. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum IL-1β than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

Experiment 3D: Effects of Maternal Separation and 1 LPS Injection on TNF-α

One LPS injection significantly increases serum TNF-α levels in males

A 2x2 ANOVA was used to determine if maternal separation impacted serum TNF-α levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 35)= 103.325$, $p <$ .001) such that LPS-treated animals had significantly more serum TNF-α than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 35)= .808$, *NS*) or a significant Condition x Treatment interaction ($F(1, 35)= .823$, *NS*); Figure 28). Overall, LPS, but not maternal separation, significantly increased serum TNF-α levels.

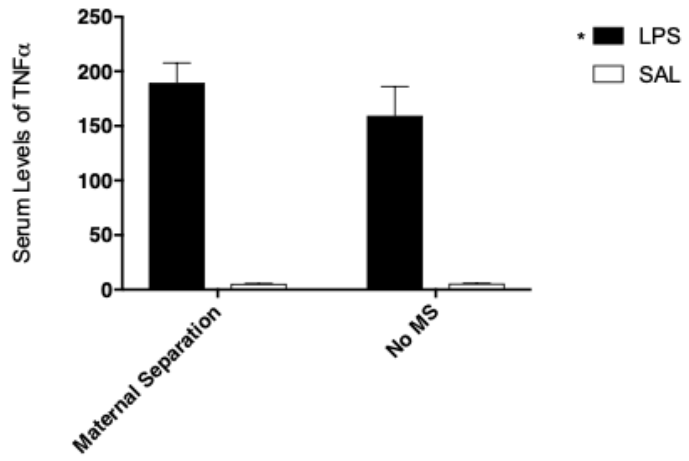

Figure 28. Maternal separation did not significantly impact serum TNF-α levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum TNF-α than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

One LPS injection significantly increases serum TNF-α levels in females

A 2x2 ANOVA was used to determine if maternal separation impacted serum TNF-α levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 36)= 50.411$, $p < .001$) such that LPS-treated animals had significantly more serum TNF-α than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 36)= .075$, *NS*) or a significant Condition x Treatment interaction ($F(1, 36)= .057$, *NS*); Figure 29). Overall, LPS, but not maternal separation, significantly increased serum TNF-α levels.

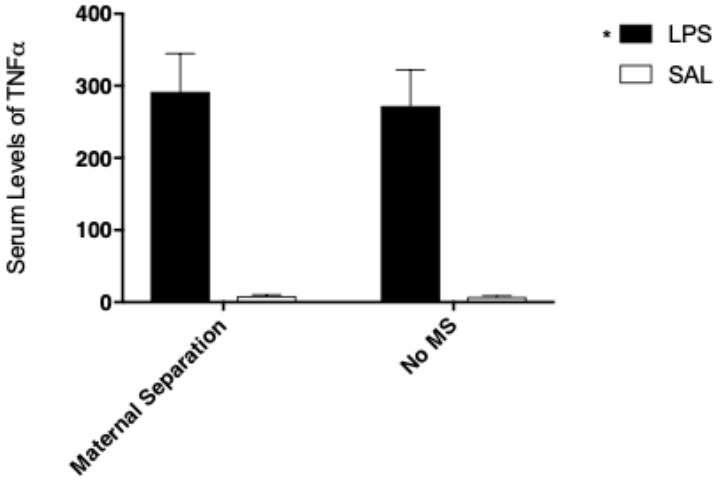

Figure 29. Maternal separation did not significantly impact serum TNF-α levels in females. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum TNF-α than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

Experiment 3E: Effects of Maternal Separation and 1 LPS Injection on IL-10

One LPS injection significantly increases serum IL-10 levels in males

A 2x2 ANOVA was used to determine if maternal separation impacted serum IL-10 levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 36)= 172.830$, $p <$ .001) such that LPS-treated animals had significantly more serum IL-10 than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 36)= .839$, *NS*) or a significant Condition x Treatment interaction ($F(1, 36)= .815$, *NS*); Figure 30). Overall, LPS, but not maternal separation, significantly increased serum IL-10 levels.

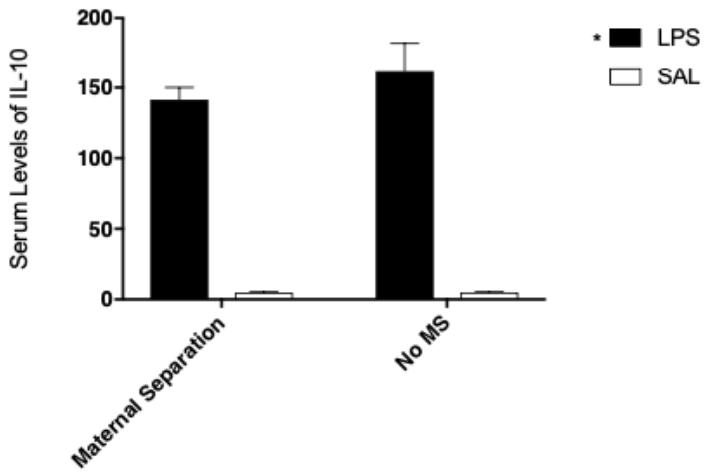

Figure 30. Maternal separation did not significantly impact serum IL-10 levels in males. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum IL-10 than saline animals regardless of condition. Significance differences (p<0.05) are designated by *.

One LPS injection significantly increases serum IL-10 levels in females

A 2x2 ANOVA was used to determine if maternal separation impacted serum IL-10 levels. Analyses revealed a main effect of Treatment (LPS or saline; $F(1, 33)= 222.609$, $p <$ .001) such that LPS-treated animals had significantly more serum IL-10 than saline-treated animals. There was not a significant main effect of Condition (MS or no MS; $F(1, 33)= .348$, *NS*) or a significant Condition x Treatment interaction ($F(1, 33)= .402$, *NS*); Figure 31). Overall, LPS, but not maternal separation, significantly increased serum IL-10 levels.

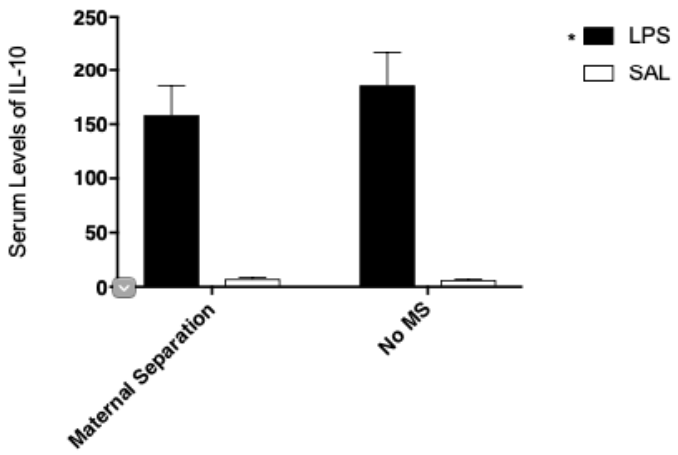

Figure 31. Maternal separation did not significantly impact serum IL-10 levels in females. A 2x2 ANOVA revealed a main effect of treatment such that LPS animals had significantly more serum IL-10 than saline animals regardless of condition. Significance differences ($p<0.05$) are designated by *.

Discussion

Experiment 3 aimed to investigate the interaction between early life maternal separation, and adulthood LPS induced cytokine levels, in addition to investigating potential effects of maternal separation on anxiety like behaviors. It was hypothesized that maternal separation would significant increase levels of serum IL-1β, TNF-α, and IL-10 following 1 LPS injection as compared with saline, and non-maternally separated control animals. It was also hypothesized that maternal separation would alter time spent exploring the center of the open field chamber as compared with non-maternally separated animals in open field behavioral testing. The hypotheses were partially supported.

It was hypothesized that maternal separation would alter the open field behavior of the MS mice as compared to the non-maternally separated mice. The hypotheses were supported. The open field data revealed maternally separated males spent significantly less time in the center of the area and travelled more distance than did non-maternally separated males. This was

consistent with a study that found that prenatal stress in rats led to increased distance travelled and more time spent in the corners, not the center, of the open field chamber (Vallee et al., 1997). Conversely, maternally separated females spent more time in the center than did non-maternally separated females. This seems counterintuitive, as females have a greater prevalence of mood and anxiety disorders than males (Altemus et al., 2014); however multiple studies of early life stress have demonstrated that it leads to no change or reduced anxiety behaviors in females (Murthy & Gould, 2018; Slotten et al., 2006; Lehmann et al., 1999; McIntosh, Anisman, & Merali, 1999). Additionally, Romeo et al. (2003) also found that maternally separated C57BL/6 males displayed higher levels of anxiety behaviors while maternally separated females displayed reduced anxiety in open field testing. However, these effects are not universal, as Wongwitdecha, Yoopan, & Srisomboonlert (2008) found maternally separated rats showed hyperlocomotion and more exploration, while Tsuda and Ogawa (2012) found maternally separated females spent less time in the center during open field testing. A potential reason for these inconsistencies is that researchers have shown that the effects of maternal separation on later open field behavior may be timing-dependent, as maternal separation from post natal day 2–15 led to decreased time spent in center in open field testing, but not in animals maternally separated on post natal day 7–20 (Roque et al., 2014). The effects of maternal separation on anxiety behavior later in life is likely parameter-dependent, one such parameter being the onset of stress, and this has contributed to mixed results in the literature. The data also partially fit with a trend in the recent literature indicating females may be more resilient to the hippocampal effects of childhood maltreatment, but not to the resultant anxiety related psychopathology (Loi et al., 2017). Other factors that may influence the results include that differences may exist

across species and even strain, and that females may behave differently depending on their estrous cycle (Romeo et al., 2003).

It was also hypothesized that maternally separated animals would have higher levels of proinflammatory cytokines following 1 LPS injection than non-maternally separated animals. This hypothesis was not supported. As hypothesized, following 1 LPS injection, LPS-treated males and females displayed increased levels of IL-1β, TNF-α, and IL-10 as compared with saline-treated animals. However, maternal separation did not further increase the levels of these cytokines. This is consistent with previous research demonstrating that the cytokines IL-1β and TNF-α were not exacerbated by maternal separation following inflammation (Genty et al., 2018). However, this is inconsistent with Pinheiro et al. (2014), demonstrated that maternal separation increased the levels of IL-10 and TNF-a in the hippocampus. In the present experiment, there may be an alteration in central cytokines, just not in peripheral cytokines. Another possibility is that there is a ceiling effect with the LPS dose, where 250 μg/kg elevates cytokine production more than can be altered by LPS, but there would be a difference between conditions with a smaller dose. Additionally, it may be that it is due to the onset or duration of the maternal separation, and may be time-dependent.

CHAPTER 5: GENERAL CONCLUSION

The number of new cases of Alzheimer's disease and other dementias annually is projected to double by 2050, and the estimated disease prevalence in the United States is expected to reach nearly 14 million by 2050. Healthcare payments for people afflicted with AD and other dementias was estimated to be 277 billion dollars in 2018 ("2018 Alzheimer's Disease Facts and Figures," 2018). With so many individuals and families affected by Alzheimer's disease, understanding the pathology and its causes is crucial. There are still many aspects of the disease yet to be elucidated, including how stress interacts with and impacts disease pathogenesis. One such stressor is early life stress, which can have lasting impacts into adulthood and old age, and thus potentially affect Alzheimer's disease. The present studies sought to investigate the relationship between maternal separation, an early life stressor, and Alzheimer's disease like pathology in adulthood.

The decision to explore these factors in non-transgenic animals had several bases. Research studying the extent to which transgenic animals model human AD has generally concluded there are significant shortcomings in the translational validity of the research (Platt et al., 2013). Additionally, transgenic AD animals model genetic, or early-onset, AD in humans. The vast majority of Alzheimer's cases are patients suffering from sporadic AD, with onset after the age of 65, and without the clear genetic causes of early-onset AD. Previous research established a model of AD-like pathology in non-transgenic animals that is inflammation-based, and potentially more similar to sporadic AD than transgenic animals. The present experiments utilized non-transgenic C57BL/6J mice administered LPS to induce AD-like pathology. It was hypothesized that maternal separation would exacerbate these LPS-induced markers, specifically that maternal separation would lead to increased AD-like markers, proinflammatory cytokines,

hippocampal amyloid beta, and BACE1 expression following LPS injections. It was also hypothesized that maternal separation would lead to cognitive deficits in adulthood accompanied by decreases in hippocampal proBDNF expression.

In Experiment 1, following 7, once-daily injections, contextual fear conditioning data revealed cognitive impairment of maternally separated males over non-maternally separated males. Additionally, only maternally separated males had increased hippocampal BACE1 expression, indicating potential sex specificity in the response to the stress. Experiment 2 showed that maternal separation led to a cognitive impairment in contextual fear conditioning, concurrent with decreased hippocampal proBDNF expression, in male and female animals. Additionally, maternally separated females had increased hippocampal Aβ after 3 LPS injections. These studies displayed that maternal separation can impact adulthood Alzheimer's-like pathologies in both males and females in a sex-specific manner. Finally, Experiment 3 demonstrated that early life maternal separation alters anxiety behavior in adulthood, as the open field data revealed maternally separated males spend significantly less time in the center of the area and travelled more distance than did non-maternally separated males. Conversely, maternally separated females spent more time in the center than did non-maternally separated females. This demonstrates that maternal separation impacts anxiety like behaviors in adulthood in both males and females. Taken together, these results indicate that maternal separation in C57BL/6J mice does impact some facets of AD-like pathology later in life, and it appears to be in a sex specific manner. Further research is needed in order to more fully explore the mechanisms behind these effects.

These present experiments examine the interaction between an early life stressor and inflammation induced Aβ production later in life. These studies more closely examined AD-

related markers in non-transgenic mice in conjunction with LPS administration, contributing to our understanding of what factors impact sporadic AD pathology. As research has investigated early life stress and AD pathology in AD transgenic and non-transgenic mice, the current research can be situated between these two alternatives. Future research will explore the mechanisms behind the interaction between maternal separation and adulthood AD-like markers. In order to investigate potential mechanisms, extensions of this research should examine the role of the HPA axis and potential epigenetic modifications through quantifying glucocorticoid receptor (GR) and DNA methyltransferase 1 (DNMT1) levels, as research has demonstrated decreased HPA axis regulation can potentiate hippocampal damage due to Aβ (Friedler et al., 2015) and stress can play a role in this (Solas et al., 2010). HPA axis hyperactivity has also been shown to cause decreased cell number in the hippocampus due decreased cell proliferation and increased apoptotic death as well as decreases in synaptophysin and PSD-95 following maternal separation, further potential mechanistic factors (Martisova et al., 2013). Moreover, the link between gliosis and inflammation should be explored by examining microglial activation and central pro-inflammatory cytokine expression, as it is possible that stress induced-gliosis results in an increase in microglial production of pro-inflammatory cytokines which can contribute toward the exacerbation of Aβ (Walker et al., 2013; Weber et al., 2015).

In summary, our findings were consistent with research demonstrating that early life stress can have adverse impacts in adulthood. We extended prior research in showing that maternal separation can significantly impact Alzheimer's like markers, as it impaired cognition and decreased proBDNF expression in males and females. There was also an interaction effect where maternally separated females who got 3 LPS injections had increased hippocampal Aβ, and maternally separated males had a marginally significant increase in BACE1 expression after

7, once-daily injections. These findings demonstrate the need for further research exploring how early life stress can impact patients later in life, and how it may interact with Alzheimer's disease. As the prevalence of Alzheimer's disease and the aging population continue to grow, answers to these questions become increasingly imperative.

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VITA

PERSONAL BACKGROUND

Julia Lauren Peterman
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EDUCATION

Diploma, Ursuline Academy of Dallas, Dallas, Texas, 2011
Bachelor of Science, Psychology, Xavier University, Cincinnati, Ohio, 2015
Bachelor of Arts, History, Xavier University, Cincinnati, Ohio, 2015
Master of Science, Experimental Psychology, Texas Christian University, 2018
Doctor of Philosophy, Experimental Psychology, Texas Christian University, 2020

EXPERIENCE

Supplemental Instructor in Anatomy and Physiology, Learning Assistance Center, Xavier University, 2013-2015
Tutor in Anatomy and Physiology, Psychology, and History, Learning Assistance Center, Xavier University, 2013-2015
Research Assistant, Department of Psychology, Xavier University, 2013-2014
Research Assistantship, Department of History, Xavier University, 2014
Teaching Assistantship, Texas Christian University, 2015-present

VOLUNTEER WORK

Site Leader for Xavier University volunteers to Evanston Academy Elementary School, 2012, 2014-2015

PROFESSIONAL MEMBERSHIPS

Golden Key Honors Society, 2017-present
Psychoneuroimmunology Research Society, 2017-present
Society for Neuroscience, 2015-present
Phi Beta Kappa Honors Society, 2015-present
Alpha Epsilon Delta Pre Health Professional Honors Society, 2014-present
Alpha Sigma Nu Jesuit Honors Society, 2014-present
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ABSTRACT

EFFECTS OF MATERNAL SEPARATION ON ALZHEIMER'S DISEASE-RELATED PATHOLOGY IN ADULT C57BL/6J MICE

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Alzheimer's Disease (AD) is the most common form of dementia, with an incidence expected to drastically increase as our population grows older. AD is characterized by the accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles. Our laboratory has previously demonstrated that 7 consecutive daily injections of LPS (250 μg/kg; i.p.) result in increased inflammation and significant elevation of amyloid-beta within the hippocampus of C57BL/6J mice. Given the relationship between stress, inflammation, and AD pathology, we sought to explore how an early life stressor, maternal separation (MS), could impact AD-like markers in adulthood. Mouse pups were separated from their mothers for three hours daily from post-natal day 2 (PND2) to PND21 and then were allowed to age in standard housing conditions into adulthood. At 4–6 months of age, mice received LPS or Saline injections and cognition was assessed utilizing a contextual fear conditioning (CFC) and open field paradigms. Tissue was collected and hippocampal Aβ levels were quantified via ELISA, while western blotting was utilized to explore potential mechanisms behind Aβ alterations. Maternal separation significantly impaired cognitive function in CFC and resulted in decreased hippocampal BDNF expression in males and females. MS also exacerbated LPS-induced accumulation of Aβ in females, and increased hippocampal BACE1 expression in male mice. MS also altered anxiety behavior in open field in a sex-specific manner. Overall, the results suggest that early-life stress can exacerbate inflammation-induced AD-like pathologies in adulthood. Understanding how the

interaction of stress and immune function may increase one's potential risk for AD, as well as impact AD pathogenesis, are some of the first steps in developing effective interventions.